

IDENTIFICATION OF ETIOLOGY OF AUTISMBackground of the InventionField of the Invention

[0001] The invention relates to identification of etiology of autism.

Description of the Related Art

[0002] Autism is a developmental disorder, which manifests itself during early childhood. In the autistic child, communications and social interactions, are severely impaired. Unable to learn from the natural environment as most children do, the child with autism generally shows little interest in the world or people around him. Although some children with autism develop normally and even acquire advanced skills, most exhibit a wide range of behavioral problems. In reality, autism affects the way a person comprehends, communicates and relates to others. Autism was originally thought to be primarily a psychiatric condition. However, further investigation showed that genetic and environmental factors are implicated in the pathogenesis of autism (1-8). The effects of environmental factors such as infections and toxic chemicals on gene expression result in biochemical, immunological and neurological disorders found in children with autism.

Diagnosis of autism

[0003] Because we have no definitive diagnostic tests for the biological manifestations of autism, it remains one of the only neurological disorders that must be diagnosed almost entirely through behavioral symptoms. We know that autism interferes with the normal development of the brain in the areas of reasoning, social interaction, communication skills and emotions such as love and empathy. Children and adults with autism typically have deficiencies in verbal and nonverbal communication, social interactions, and leisure or play activities. Autistic people may exhibit repeated body movements such as hand flapping, rocking, or spinning; they may have unusual responses to people or attachments to objects; and they may resist changes in routines. In some cases they may exhibit aggressive or self-injurious behavior.

[0004] According to the DSM-IV or *Diagnosis and Statistical Manual for Mental Disorders, 4th* edition, published by the American Psychiatric Association (9), autism is classified as a Pervasive Developmental Disorder (PDD) characterized by twelve diagnostic criteria. These criteria fall into three categories - impairments in social interaction, impairments in communication, and a restricted repertoire of activities and interests. A diagnosis of autism requires that a child display at least six of these twelve symptoms, with a minimum number in each category.

DSM IV Diagnostic criteria for autism

Diagnosis criteria for 299.00 Autistic Disorder

[0005] A. A total of six (or more) items from (1), (2), and (3), with at least two from (1), and one each from (2) and (3):

1. qualitative impairment in social interaction, as manifested by at least two of the following:

- a. marked impairment in the use of multiple nonverbal behaviors such as eye-to-eye gaze, facial expression, body postures, and gestures to regulate social interaction
- b. failure to develop peer relationships appropriate to developmental level
- c. lack of spontaneous seeking to share enjoyment, interests, or achievements with other people (e.g., by a lack of showing, bringing, or pointing out objects of interest)
- d. lack of social or emotional reciprocity

2. qualitative impairments in communication as manifested by at least one of the following:

- a. delay in, or total lack of, the development of spoken language (not accompanied by an attempt to compensate through alternative modes of communication such as gesture or mime)
- b. in individuals with adequate speech, marked impairment in the ability to initiate or sustain a conversation with others
- c. stereotyped and repetitive use of language or idiosyncratic language

- d. lack of varied, spontaneous make-believe play or social imitative play appropriate to developmental level
- 3. restricted repetitive and stereotyped patterns of behavior, interests and activities, as manifested by at least one of the following:
 - a. encompassing preoccupation with one or more stereotyped and restricted patterns of interest that is abnormal either in intensity or focus
 - b. apparently inflexible adherence to specific, nonfunctional routines or rituals
 - c. stereotyped and repetitive motor mannerisms (e.g., hand or finger flapping or twisting, or complex whole-body movements)
 - d. persistent preoccupation with parts of objects

[0006] B. Delays or abnormal functioning in at least one of the following areas, with onset prior to age 3 years: (1) social interaction, (2) language as used in social communication, or (3) symbolic or imaginative play.

[0007] C. The disturbance is not better accounted for by Rett's Disorder or Childhood Disintegrative Disorder.

[0008] If a child does not fit the definition of autism given above, he or she may be diagnosed with a condition called Pervasive Developmental Disorder Not Otherwise Specified (PDD-NOS). Such a diagnosis of non-specific forms of Pervasive Developmental Disorder (PDD) may include atypical types of autism that do not fall into the above categories because of late age of onset, for example, or subthreshold or atypical symptoms. According to the DSM-IV, this diagnosis is to be used when autistic-like behaviors are present - in particular, when there is severe impairment in the development of social and verbal communication skills - but the child does not meet the criteria for classic autism or any other specific Pervasive Developmental Disorder, Schizophrenia, Schizotypal Personality Disorder or Avoidant Personality Disorder (9).

Summary of the Invention

[0009] An embodiment provides for a method for determining etiology of an autistic spectrum disorder in a patient, comprising the steps of:

a) determining a level of at least one infectious agent derived antigen or antibody against an infectious agent derived antigen, at least one toxic chemical derived antigen or an antibody against a toxic chemical, and at least one dietary protein derived antigen or antibody against a dietary protein, in one or more samples from the patient;

b) comparing the level of antigens and/or antibodies determined in step a) with a normal level of the antigens and/or antibodies from control subjects, wherein

(i) normal level or lower than normal level of antigens and/or antibodies for the each of said antigens indicate absence of an etiology of autistic spectrum disorder from presence of said antigens; and

(ii) higher than normal level of antigens and/or antibodies for one or more of said antigens and/or antibodies indicates a likelihood of the autistic spectrum disorder being based on the presence of said antigens.

[0010] Another embodiment provides for a method for determining etiology of an autistic spectrum disorder in a patient, comprising the steps of:

a) determining a level of antibodies to a self-tissue or peptide in one or more samples from the patient; and

b) comparing the level of antibodies determined in step a) with a normal level of the antibodies from control subjects, wherein

(i) normal level or lower than normal levels of antibodies indicate absence of etiology of autistic spectrum disorder from presence of said antibodies; and

(ii) higher than normal level of the antibodies indicates a likelihood of the autistic spectrum disorder being based on the presence of said antibodies.

Brief Description of the Drawings

[0011] Figure 1 illustrates induction of neuroimmune disorders by infections, toxic chemicals, and dietary proteins or peptides in autism.

[0012] Figure 2 illustrates the role of endogenous opioids and their receptors on immune and brain functions.

[0013] Figure 3 illustrates dietary peptides binding to μ , γ , and κ opioid receptors on lymphocytes and on the cells of the central, peripheral and autonomic nervous system resulting in abnormal neuroimmune communications, brain and immune functions.

[0014] Figure 4 illustrates cellular and humoral immune mechanisms in infections- and xenobiotics-induced neurotoxicity, which includes neuronal degeneration, secondary demyelination, and possibly reactive astrogliosis. Under pathological conditions, pre-existing autoreactive T-cells are generated by molecular mimicry as a result of sequence homologies or matched motifs between autoantigen and viral, bacterial, or parasitic proteins. Increased ICAM on endothelial cells by xenobiotics and bacterial toxins may allow transmigration of these auto-reactive T-cells across the blood-brain barrier resulting in cellular and humoral immune responses against nerve cells.

[0015] Figure 5 shows sequences for Dipeptidyl Peptidase IV during intestinal differentiation which is also useful in assays of preferred embodiments.

[0016] Figure 6 illustrates xenobiotics, bacterial toxins, and dietary peptides binding to DPP IV, formation of Hapten Carrier Effect, and production of antibodies against DPP IV, xenobiotics, peptides and bacterial toxins. This may result in dysfunction of DPP IV molecule and accumulation of peptides in the GI tract and in circulation.

[0017] Figure 7 shows a scattergram of serum titer of IgG antibody against different neurologic antigens (1-MBP, 2-MAG; 3-GM1; 4-SULF; 5-CONSO4; 6-MOG; 7-b-CRYS; 8-NAFP; 9-TUBULIN) and their cross-reactive peptides (10-CPP; 11-STM6P; 12-MILK-BTN) in healthy control subjects (40) and patients with autism (40) expressed as optical density in ELISA test.

[0018] Figure 8 shows percent elevation in IgG antibody against neurologic antigens and their cross-reactive peptides in healthy control subjects (40) and patients with autism (40) at cut-off point of 0.30 O.D.

[0019] Figure 9 shows a scattergram of serum titer of IgM antibody against different neurologic antigens (1-MBP, 2-MAG; 3-GMI; 4-SULF; 5-CONSO4; 6-MOG; 7-b-CRYS; 8-NAFP; 9-TUBULIN) and their cross-reactive peptides (10-CPP; 11-STM6P; 12-

MILK-BTN) in healthy control subjects (40) and patients with autism (40) expressed as optical density in ELISA test.

[0020] Figure 10 shows percent elevation in IgM antibody against neurologic antigens and their cross-reactive peptides in healthy control subjects (40) and patients with autism (40) at cut-off point of 0.30 O.D.

[0021] Figure 11 shows a scattergram of serum titer of IgA antibody against different neurologic antigens (1-MBP, 2-MAG; 3-GMI; 4-SULF; 5-CONSO4; 6-MOG; 7-b-CRYS; 8-NAFP; 9-TUBULIN) and their cross-reactive peptides (10-CPP; 11-STM6P; 12-MILK-BTN) in healthy control subjects (40) and patients with autism (40) expressed as optical density in ELISA test.

[0022] Figure 12 shows percent elevation in IgA antibody against neurologic antigens and their cross-reactive peptides in healthy control subjects (40) and patients with autism (40) at cut-off point of 0.30 O.D.

[0023] Figure 13 shows a scattergram of serum titer of IgG, IgM, and IgA antibodies against Dipeptidyl peptidase IV (CD26) in healthy controls subjects ◆ and autistic patients ■ and CD69 in healthy control subjects ● and autistic patients ▲ expressed as optical density in ELISA test.

[0024] Figure 14 shows a scattergram of serum titer of IgG, IgM, and IgA antibodies against gliadin peptides in healthy controls subjects ◆ and autistic patients ■ and CD69 in healthy control subjects ● and autistic patients ▲ expressed as optical density in ELISA test.

[0025] Figure 15 shows a scattergram of serum titer of IgG, IgM, and IgA antibodies against streptokinase in healthy controls subjects ◆ and autistic patients ■ and CD69 in healthy control subjects ● and autistic patients ▲ expressed as optical density in ELISA test.

[0026] Figure 16 shows a scattergram of serum titer of IgG, IgM, and IgA antibodies against dipeptidylpeptidase IV (DPP IV) in healthy, young control subjects ◆, autistic patients ■, in healthy, older control subjects ▲, and patients with autoimmune disease expressed as optical density in ELISA test.

[0027] Figure 17 shows a scattergram of serum titer of IgG, IgM, and IgA antibodies against DPPI in healthy, young control subjects ♦, autistic patients ■, in healthy, older control subjects ▲ and patients with autoimmune disease expressed as optical density in ELISA test.

[0028] Figure 18 shows a scattergram of serum titer of IgG, IgM, and IgA antibodies against CD13 in healthy, young control subjects ♦, autistic patients ■, in healthy, older control subjects ▲ and patients with autoimmune disease ● expressed as optical density in ELISA test.

[0029] Figure 19 shows a scattergram of serum titer of IgG, IgM, and IgA antibodies against gliadin peptide in healthy, young control subjects ♦, autistic patients ■, in healthy, older control subjects ▲ and patients with autoimmune disease ● expressed as optical density in ELISA test.

[0030] Figure 20 shows a scattergram of serum titer of IgG, IgM, and IgA antibodies against HSP 60 in healthy, young control subjects ♦, autistic patients ■, in healthy, older control subjects ▲ and patients with autoimmune disease ● expressed as optical density in ELISA test.

[0031] Figure 21 shows percent positive sera from patients with autism for IgA □, IgG ▣, and IgM ■ antibodies against gliadin and HSP 60 peptides, which are positive for DPP IV, DPP I, or CD13 Antibodies.

[0032] Figure 22 shows percent positive sera from patients with autoimmune disease for IgA □, IgG ▣, and IgM ■ antibodies against gliadin and HSP 60 Peptides, which are positive for DPP IV, DPP I, or CD13 Antibodies.

Detailed Description of the Preferred Embodiment

[0033] Similar to many complex diseases (10), genetic and environmental factors including infections, xenobiotics, dietary proteins and peptides, play a role in the development of autism. The effects of environmental factors on genetic makeup can result in immune, gastrointestinal, neurological, biochemical and neuroimmunological abnormalities. Based on extensive research (11-15), we postulated that autism is induced by infectious agent antigens, toxic chemicals or dietary proteins. This process begins in the gastrointestinal tract

but manifests itself in the brain (Fig. 1). These factors will be explained in detail in the following sections.

[0034] An embodiment provides for a method for determining etiology of an autistic spectrum disorder in a patient, comprising the steps of:

- a) determining a level of at least one infectious agent derived antigen or antibody against an infectious agent derived antigen, at least one toxic chemical derived antigen or an antibody against a toxic chemical, and at least one dietary protein derived antigen or antibody against a dietary protein, in one or more samples from the patient;
- b) comparing the level of antigens and/or antibodies determined in step a) with a normal level of the antigens and/or antibodies from control subjects, wherein
 - (i) normal level or lower than normal level of antigens and/or antibodies for the each of said antigens indicate absence of an etiology of autistic spectrum disorder from presence of said antigens; and
 - (iii) higher than normal level of antigens and/or antibodies for one or more of said antigens and/or antibodies indicates a likelihood of the autistic spectrum disorder being based on the presence of said antigens.

[0035] Another embodiment provides for a method for determining etiology of an autistic spectrum disorder in a patient, comprising the steps of:

- a) determining a level of antibodies to a self-tissue or peptide in one or more samples from the patient; and
- b) comparing the level of antibodies determined in step a) with a normal level of the antibodies from control subjects, wherein
 - (i) normal level or lower than normal levels of antibodies indicate absence of etiology of autistic spectrum disorder from presence of said antibodies; and
 - (ii) higher than normal level of the antibodies indicates a likelihood of the autistic spectrum disorder being based on the presence of said antibodies.

Preferably, the higher than normal level of antibodies is calculated by taking a mean of levels of antibodies in individuals without symptoms relating to autistic spectrum disorder. Preferably, the higher than normal levels of antibodies is higher than about two standard deviations of normal level of antibodies of a control group.

[0036] Preferably, the determining the level of antibodies is accomplished using an immunoassay, such as ELISA, RAST, dot blot, Western blot, and ELISPOT. Preferably, the antibodies used in the immunoassay is selected from IgG, IgA, or IgM.

[0037] As used herein, "autistic spectrum disorder" refers to a developmental disorder that affects many aspects of a child's functioning. Autistic spectrum disorder can include, but is not limited to, autism, pervasive developmental disorder, and Asperger's Syndrome. Autistic spectrum disorder can occur in combination with other disorders, such as Attention Deficit Hyperactivity Disorder (ADHD) (which is part of the pervasive developmental disorder), learning disabilities (LD), anxiety disorders, obsessive-compulsive disorders (OCD), epilepsy, or mental retardation.

[0038] As used herein, "derived" or "derivative" refers to anything obtained or deduced from another.

The Role Of Infectious Agents In Autism

[0039] Many infectious agents, including Streptococcus, measles, Rubella, Cytomegalovirus, Varicella zoster, Herpes type-6 and others have long been suspected as etiologic factors in autism (2-4, 16-18). Maternal or post-maternal exposure to these infectious agents may result in neurological disorders including autism. Using the observation that maternal infection increases the risk of schizophrenia and autism in offspring, recently it has been shown that respiratory infection of pregnant mice (both BALB/c and C57BL/6 strains) with the human influenza virus resulted in offspring that displayed highly abnormal behavioral responses as adults. As in schizophrenia and autism, these offspring displayed deficits in prepulse inhibition (PPI) in the acoustic startle response. Compared with control mice, the infected mice also showed striking responses to the acute administration of antipsychotic and psychomimetic drugs. Moreover, these mice were deficient in exploratory behavior in both open-field and novel-object tests, and they were

deficient in social interaction. At least some of these behavioral changes were likely attributable to the maternal immune response itself. They concluded that abnormal levels of cytokine production, which interfere with neuroimmuno-communications, are responsible for abnormal development of the brain (19-20).

[0040] Another explanation for disease development postulates that specific antigenic epitopes from an unspecified infectious agent or agents induce(s) a host immune response in which cross-reactivity with myelin triggers disease, a concept referred to as molecular mimicry. In this scenario, certain T-cells and/or antibodies elicited in response to antigens of the infectious agent also recognize relevant self-antigens in the CNS, thereby initiating the destructive autoimmune process (11-15, 21-27).

Infectious Agents and Response to Vaccinations

[0041] Many infectious agents, including measles, rubella virus and Cytomegalovirus, Herpes Type-6 and anaerobe bacteria such as *Clostridium difficile* have been implicated in autism. Therefore, the detection of nucleic acids and antibodies in blood may indicate ongoing infection and justify treatment with anti-bacterial or anti-viral agents (77-80). Moreover, measurements of antibodies against measles, mumps, rubella (MMR), diphtheria, pertussis, tetanus (DPT) and Hepatitis B will assess immune response to immunization and production of protective antibodies. Moderate elevation in IgG antibody against the components of MMR, DPT and Hepatitis B vaccines indicate optimal immune response and good immunological memory to these bacterial and viral antigens. High or very high levels of IgG antibodies against antigenic components of the vaccines indicate overactive immune response against them. Low levels or absence of IgG antibodies against components of vaccines may indicate lack of immunological memory and possibly immune deficiency in the immunized individual.

[0042] Examples of antibodies associated with infectious agents and response to vaccinations to be tested include, but are not limited to, measles, mumps, rubella, diphtheria toxoid, pertussis, tetanus toxoid, hepatitis B, herpes type 6, and clostridium neurotoxin.

The Role Of Heavy Metals And Other Toxic Chemicals In Autism

[0043] Xenobiotics or toxic chemicals have been suspected to contribute to the induction of autoimmunity (30-34). Many environmental chemicals or drugs are toxic to hosts, and their detoxification is achieved primarily in the liver. During their metabolism, they may form reactive metabolites, which can then modify cellular proteins to form neoantigens. The precise mechanisms that lead to modification of self-proteins and the molecular requirements for this modified self to induce tolerance breakdown remain to be established. However, it is important to note that the direct toxic effect of xenobiotics is usually dose dependent and may be evident in the majority of individuals shortly after drug intake; hence, they are relatively easy to identify. In contrast, the immune-mediated effects that follow the intake of drugs or xenobiotics may take a prolonged period of time to be clinically manifest, making the identification of the causative agents a formidable task (35).

[0044] Edelson and Cantor (5, 36) demonstrated that neurotoxicants play a possible role in more than 90% of autistic children. These authors presented evidence for genetic and environmental aspects of a proposed process involving immune system injury and autoimmune responses secondary to exposure to immunotoxins. They believe that activation of the immune system is caused by toxicants leading to the production of autoantibodies against haptens, i.e., the toxic chemicals attached to brain proteins. The subsequent damage may be considered a component in the etiologic process of neurotoxicity in the autistic spectrum.

[0045] For a chemical compound to lead to an autoimmune response, it is generally thought that the compound must first become covalently bound to a carrier protein (37, 39). Immune reactions to drugs or their metabolites can develop when a hapten carrier complex interacts with gut-associated lymphoid tissues (GALT) that constitute the largest lymphoid organ (38). If covalent adducts of drugs or other chemical compounds are formed in GALT, it seems reasonable that they may lead to immune responses and chemically-induced Type I- Type IV allergic reactions (37). In fact, the non-steroidal anti-inflammatory Diclofenac has been shown to cause a variety of idiosyncratic adverse reactions such as hemolytic anemia, hepatotoxicity, agranulocytosis, and anaphylaxis, all of which are components of immune reactions to protein adducts of Diclofenac (37-39). These protein adducts can be formed by direct reaction with tissue antigens or cytochrome P450

dependent and UDP-glucuronosyltransferase dependent pathways of metabolism. For example, immunoblot analysis of small intestine homogenates and isolated enterocytes with drug-specific antiserum revealed protein adducts of diclofenac. Two of these adducts of Diclofenac were identified as aminopeptidase N (CD 13) and sucrase-isomaltase (38). Intestinal protein adducts of chemicals can, therefore, be formed in GALT where they may lead to allergic reactions, inflammation and autoimmunity.

[0046] Among many toxicants, such as thimerosal, merthiolate, ethyl mercury, or other mercury-based compounds, in vaccines has been associated with immune injuries described in children with autism (41-44). Contrary to many haptens that bind covalently to a single amino acid, such as lysine, metal complexes consist of a central metal ion composed of four different amino acids, and hence they possess increased complex stability (37). To demonstrate possible binding of ethyl mercury to DPP IV and CD69, we postulated that in addition to infectious agent antigens such as Streptokinase, ethyl mercury (xenobiotic) binds to different lymphocyte receptors and tissue antigens. We assessed this hypothesis first by measuring IgG, IgM and IgA antibodies against CD26, CD69 and SK against ethyl mercury bound to human serum albumin in patients with autism. A significant percentage of children with autism developed anti-SK, and anti-ethyl mercury antibodies, concomitant with the appearance of anti-CD26 and anti-CD69 autoantibodies. These antibodies are synthesized as a result of SK and ethyl mercury binding to CD26 and CD69, indicating that they are specific. Immune absorption demonstrated that only specific antigens, like CD26, were capable of significantly reducing serum anti-CD26 levels. However, for direct demonstration of SK and ethyl mercury binding to CD26 or CD69, microtiter wells were coated with CD26 or CD69 alone or in combination with SK or ethyl mercury and then reacted with enzyme labeled rabbit anti-CD26 or anti-CD69. Adding these molecules to cD26 or CD69 resulted in 28-86% inhibition of CD26 or CD69 binding to anti-CD26 or anti-CD69 antibodies. We, therefore, propose that bacterial antigens and thimerosal (ethyl mercury) in individuals with pre-disposing HLA molecules, bind to CD26 or CD69 and induce antibodies against these molecules as well as to lymphocyte receptors and tissue antigens, resulting in autoimmune reaction in children with autism.

Neuroimmune Abnormalities Induced By Xenobiotics And Metals

[0047] It is of considerable interest that antibodies to neuron-specific antigens are prevalent in populations exposed to environmental and occupational chemicals and in patients with neurodegenerative diseases in which viruses or other infectious agents are the suspected etiological agents. For example, IgG antibodies to MBP, neuronal cytoskeletal proteins and neurofilaments are detected in workers exposed to lead or mercury (45). The titer of these antibodies is significantly correlated with blood lead or urinary mercury, which are the typical indices of exposure. Moreover, the level of these antibodies is correlated with the degree of sensorimotor deficits, because these antibodies interfere with neuromuscular function (46).

[0048] Taking into consideration the regulatory interactions between the nervous system and the immune system, as well as the detection of MBP and NAEP autoantibodies, it is therefore quite plausible to propose that drugs and environmental toxins might have detrimental effects on neuroendocrine-immune circuits, thereby resulting in autism. Toxic chemical exposure to substances, such as polychlorinated biphenyl, mercury, lead and other similar potentially harmful agents may induce alteration or over-expression of the genes involved in regional brain glial fibrillary acidic protein (GFAP) and astroglial glucose regulated protein (GRP). The astroglial cytoskeletal element GFAP, neurotypic and gliotypic proteins or neurofilament triplet are generally accepted as sensitive indicators of neurotoxic effects in mature brains (47, 48).

[0049] Over-expression of the gene results in altering the structural differentiation of astrocytes and the subsequent autoimmune response to neurofilaments and astroglial glucose regulated proteins. Autoantibodies against neurological antigens in autism have been studied in our laboratory extensively and found to be elevated in children with autism (11-15). The high prevalence of these autoantibodies in neurodegenerative and neuropsychiatric disorders has led many investigators to believe that these antibodies reflect an alteration of the blood-brain barrier, which promotes the access of immunocompetent cells to the central nervous system (49-52).

[0050] In these studies, we were able to present viable evidence in support of the genetic and environmental aspects of a hypothetical process believed to cause immune

system injury secondary to immunotoxins exposure. Activation of the immune system is caused by toxicants, leading to the production of autoantibodies against haptens -the toxic chemicals attached to brain proteins. The resulting damage may be considered a component in the etiologic process of neurotoxicity in the autistic spectrum.

Autoimmune Reaction Induced by Heavy Metals

[0051] Mercury is a widely distributed environmental and industrial pollutant. This is why methyl mercury is often detected in many fish. In fact, ethyl-mercury or thimerosal has been used in increasing amounts as preservatives in many vaccines since the 1950's. Therefore, during the first year of life when the immune system is in the process of maturation, children become exposed to up to 100 micrograms of mercury, which greatly exceeds the CDC threshold. Exposure to large doses of mercury results in acute renal tubular lesions and immunosuppression, whereas chronic administration of smaller doses can lead to development of systemic autoimmunity (31-34). The characteristic features of mercury-induced autoimmunity are very similar to manifestations of SLE. This includes:

- increased levels of Class II MHC
- antinuclear antibody production
- hypergammaglobulinemia
- polyclonal antibody to self-antigens
- formation of immune complexes
- lymphocyte proliferation
- necrotizing vasculitis

[0052] Mercury-induced autoimmunity is also similar to lupus in that the disease process requires CD4+ T-cells, T- and B-cell stimulatory molecules and interferon- γ , which strongly suggests identical pathogenic mechanisms. Given the complexity of metal interaction with cellular and subcellular components of the immune system and the large number of molecules that may be affected, genetic studies were initiated to define the genes responsible for sensitivity of resistance to mercury-induced autoimmunity. A single major quantitative trait locus on chromosome 1, designated as Hm1 was linked to glomerular immune complex deposits (81). Mercury is only one of a number of immunostimulatory

heavy metal xenobiotics that can induce adverse immunotoxicity. Several of these such as silver or gold also promote the production of anti-fibrillarin autoantibodies only in mercury-sensitive mouse strains (82).

[0053] Indeed after injection of methyl-mercury, a number of murine strains develop an antibody response against U3 small nucleolar ribonucleo-proteins called fibrillarin and chromatin. These antibodies have also been detected in humans with scleroderma. Therefore, detection of anti-nuclear antibody along with metals, fibrillarin and chromatin antibodies and elevation in immune complexes indicate involvement of metals in induction of inflammation and autoimmunity in autism (82). Further, production of these antibodies may indicate a lack of functional metallothionein at cellular level.

[0054] Examples of antibodies associated with autoimmune reaction and involvement of metals to be tested include, but are not limited to, anti-nuclear protein, mercury, fibrillarin, chromatin, immune complexes, and metallothionein.

Neuroimmune Antibodies Induced by Dietary Proteins and Infectious Agents

[0055] As mentioned above, many infectious agents have long been suspected of being etiologic factors in autism. Whether or not these viruses actually induce brain autoantibodies has not yet been explored. For this reason, we decided to review the available scientific literature and found that over sixty different microbial peptides have been reported to cross-react with human brain tissue and MBP. Furthermore, these peptides not only have the capacity to cross-react with MBP and induce T-cell response, but also are also able to induce experimental autoimmune encephalomyelitis (11, 26-30).

[0056] Among families with autistic children, it is well known that the elimination of milk from the child's diet significantly improves the patient's condition. Investigators found that an encephalitogenic T-cell response to MOG can either be induced or alternatively suppressed as a consequence of immunological cross-reactivity or molecular mimicry with the extracellular IV-like domain of milk protein butyrophilin. All of these clinical laboratory findings shed light on our detection of higher levels of antibodies against milk antigens in autistic sera. Based on earlier publications, we chose Streptococcus synthetic peptide containing the conserved M protein or brain crossreactive epitope, a

Chlamydia pneumoniae-specific peptide and the butyrophilin milk peptide, which modulates the encephalitogenic T-cell response to MOG in experimental autoimmune encephalomyelitis for our cross-reactivity study (11, 65).

[0057] Detection of IgG, IgM and IgA antibodies against myelin basic protein, neurofilaments and their cross-reactive epitopes in milk, Streptococcus and Chlamydia may justify treatment with antibiotic and/or elimination diet.

[0058] Examples of neuro-autoimmune antibodies induced by dietary proteins and infectious agents and antibodies associated with the neuro-autoimmune antibodies to be tested include, but are not limited to, myelin basic protein, neurofilament, milk butyrophilin, streptococcus M protein, and chlamydia pneumoniae.

Binding Of Dietary Peptides To Different Tissue Enzymes May Promote Development Of Peptidase Antibodies In Children With Autism

[0059] Opioid peptides are available from a variety of food sources. These dietary proteins and peptides, including casein, casomorphins, gluten (GLU) and gluteomorphins, can stimulate T-cells, induce peptide-specific T-cell responses, and abnormal levels of cytokine production, which may result in inflammation, autoimmune reactions and disruption of neuroimmune communications (54-57). In celiac disease (CD), a majority of patients who express HLA-DQ2 and/or DQ8 react to a 33-mer peptide and 15 other T-cell stimulatory peptides (58, 59). This peptide binding to HLA-DQ2 and HLA-DQ8 molecules is most efficient when negatively charged amino acids are present at anchor positions in the peptide. Yet GLU contains very few negatively charged amino acids, which makes GLU-derived peptides low affinity ligands for HLA-DQ2 and -DQ8. This paradox has been solved by finding that enzyme tissue transglutaminase, target of endomysium-specific antibodies in CD patients, can modify GLU peptides by conversion of glutamine residues into glutamic acid, which introduces negative charges favored for binding (58-60).

[0060] A majority of children with autism cannot tolerate wheat and milk proteins or peptides and hence elimination of these peptides from the diets significantly improves their conditions. This clinical finding correlates with laboratory results reported earlier by our

group in children with autism (11-15) and by different investigators in MS-like syndromes (61-64). They found that an encephalitogenic T-cell response to myelin oligodendrocyte glycoprotein (MOG) could be either induced or alternatively suppressed as a consequence of immunological cross-reactivity or "molecular mimicry" with the extra-cellular IV-like domain of milk protein called butyrophilin (BTN) (65). We detected IgG, IgM and IgA antibodies against nine specific neuron-specific antigens in the sera of children with autism. These antibodies were found to bind with different encephalitogenic molecules that have sequence homologies to a milk protein (11).

[0061] Indeed, when we tested IgG, IgM and IgA antibodies against milk peptides, we found that every single serum with ELISA values higher than 0.3 O.D. against neurological antigens also exhibited high levels of antibodies against neurological antigens and antibodies against milk peptides in a higher percentage of experimental sera. Similar to milk peptides, antibodies against different gliadin peptides have also been described in celiac disease and gluten ataxia (66-67).

Food Allergies and Intolerance

[0062] Food allergies may be said to be contributory to the behavioral disorders of individuals inflicted in autism. Sensitivity to gluten and milk are thought to be the major food allergens in these patients. In one study, nineteen children with autistic syndromes were treated with either gluten-free milk and milk-reduced diets, or milk-free and gluten-reduced diets. Before treatment, five of the fifteen fully studied patients had increased levels of IgA antibodies to casein or gluten. After following the diet for a year, improvement was noted in terms of increased social contact, decreased stereotypy, an end to self-mutilation (like head banging), and a decrease in "dreamy state" periods. These improvements were accompanied by a significant decrease in urinary peptide excretion. The possible mechanism is that children with autism suffer from one or more peptidase defects that fail to break down "exomorphins" (exogenous opioids) found in milk and wheat. These exorphin peptides then gain entry into the brain where they significantly disrupt brain chemistry (see Figures 2, 3).

[0063] The presence of other food allergies should also be determined, as food allergies are likely the factor responsible for the increased intestinal permeability noted in

these patients. In fact, increased gut permeability has been suggested as a possible causative factor for autism (73, 77).

[0064] Examples of antibodies associated with food allergy and intolerance to be tested include, but are not limited to, milk, caseomorphin, wheat gluten/gliadin, gluteomorphin, corn, soy, and tissue enzyme, such as transglutaminase which may modify resultant dietary peptides.

Cross-Reaction Between Gliadin And Cerebellar Purkinje Cells As A Possible Mechanism For Neuroimmune Abnormalities In Autism

[0065] One of the most frequent presentations of gluten sensitivity is the neurologic dysfunction called gluten ataxia. Up to 33% of patients presenting with neurologic dysfunction and 90% of patients presenting with pruritic vesicular rash of dermatitis herpetiformis associated with gluten sensitivity also have celiac disease (66). While the remaining patients have serologic markers or anti-gliadin antibodies and genetic susceptibility (HLADQ2), they do not have histologic evidence of small bowel involvement. Based on a major epidemiologic study involving more than 200 patients, gluten ataxia was found to account for 40% of cases with idiopathic sporadic cerebellar degeneration. When patients with gluten ataxia were autopsied, perivascular cuffing with inflammatory cells, predominantly affecting the cerebellum, and loss of Purkinje cells were detected. These inflammatory reactions resulting in Purkinje cell loss imply that the neurologic insult may be immune-mediated (67, 69, 70). It is not clear whether such immune-mediated damage is primarily cellular or antibody-driven. In a recent study, investigators assessed the reactivity of sera from patients with gluten ataxia, patients newly diagnosed with celiac disease without neurologic dysfunction and healthy control subjects (67).

[0066] Using indirect immunocytochemistry on human cerebellar and rat CNS tissue, cross-reactivity of a commercial IgA antigliadin antibody with cerebellar tissue was analyzed. Sera from 12 of 13 patients with gluten ataxia strongly presented stained Purkinje cells. Less intense staining was observed in some but not all sera from patients with newly diagnosed celiac disease without neurologic dysfunction. At high dilutions (1:800) staining was observed only using sera from patients with gluten, ataxia but not from control subjects.

Sera from patients with gluten ataxia also stained some brainstem and cortical neurons in rat CNS tissue. Commercial anti-gliadin antibody stained human Purkinje cells in a similar manner. Absorption of the antigliadin antibodies using crude gliadin abolished the staining in patients with celiac disease without neurologic dysfunction, but not in those with gluten ataxia. The conclusion suggested that patients with gluten ataxia have antibodies against Purkinje cells that cross-react with epitopes on Purkinje cells, and humoral immune responses are involved in the pathogenesis of gluten ataxia (67).

Direct Evidence For Structural Similarity Between Gliadin Peptides And Cerebellar Antigens

[0067] Several distinctive neurologic disorders occur in patients with paraneoplastic cerebellar degeneration (PCD). The syndrome of PCD is among the most common of these disorders and generally occurs in patients with neoplasms of the lung, breast, ovary, or with Hodgkin's disease. Neuropathologic features of PCD include extensive loss of Purkinje cells, degenerative changes in the remaining Purkinje cells, as well as variable losses of granule and basket neurons.

[0068] The presence of anti-Purkinje cell antibodies in some PCD patients suggests an autoimmune etiology. To identify the molecular targets for these autoantibodies, an Agt1.1 cDNA expression library from human cerebellum was constructed and screened with IgG from a patient with paraneoplastic cerebellar degeneration. A single clone, pCDR2, produced a fusion protein that reacted strongly with the patient's IgG. Sequencing the pCDR clones revealed 6 amino acids repeated in tandem along the entire cDNA sequence (VAL, PRO, LEU, LEU, GLU, ASP). This gene was expressed predominantly in neuroectodermal tissues (68).

Neurotransmitters and Neuroimmune Miscommunication

[0069] Autism was originally thought to be primarily a psychiatric condition. However, recent biochemical genetic studies have led to the hypothesis that the disorder is due to an organic defect in brain development. Specifically, autism is thought to be a result of abnormal serotonin metabolism in the brain. The abnormalities that have been documented include:

- abnormal release and uptake of serotonin by platelets
- abnormal kynurenine metabolism
- increased serum serotonin and free tryptophan levels
- abnormal urinary 5-hydroxyindolacetic acid (5-HIAA) levels
- abnormal urinary Serotonin metabolites

[0070] The basic defect appears to be a decrease in CNS Serotonin activity despite elevated free tryptophan levels in the serum. Abnormal serotonin metabolites seen in autistic children may significantly contribute to their mental dysfunction (83-85). Drugs such as LSD, psilocybin, ergot, and other hallucinogens are serotonin analogs, and a number of serotonin metabolites are known to be hallucinogens. It is also interesting to note that serotonin and its metabolites are produced in, and absorbed from, the intestines.

[0071] This abnormal level of serotonin along with reaction of bacterial toxins, xenobiotics and dietary peptides with different aminopeptidases and gut-neuroimmune communications results in autoantibody production against these important tissue enzymes such as somatostatin, vasoactive intestinal peptides. Formation of antibodies against peptidases results in dysfunctional enzymes and accumulation of peptides in the GI tract and in circulation. These dietary peptides in the blood may bind to G protein receptors, cause immune dysfunction and transmigrate across blood, the blood-brain barrier, and activate the local antigen-presenting cells, such as microglia and astrocytes. By reacting to μ , γ , κ opioid receptors on both lymphocytes and nerve cells, dietary peptides such as pro-dynorphins, dynorphins, casomorphins and gluteomorphins may change the level of cytokine and interfere with neuroimmune communication. Therefore, detection of high or low levels of serotonin along with antibodies to serotonin, somatostatin, vasoactive intestinal peptides, DPP IV, pro-dynorphin and dynorphin may indicate disturbance in gut-neuroimmune communication.

[0072] Examples of antibodies associated with neurotransmitters and neuroimmune miscommunication to be tested include, but are not limited to, serotonin receptor antibodies, serotonin antibodies, somatostatin antibodies, vasoactive intestinal peptide, prodynorphin, dynorphin, and dipeptidylpeptidase IV.

Pathogenesis And Mechanism Of Autoimmunity And Autism

[0073] For cross-reactive circulating antibodies to become pathogenic, they must cross the blood-brain barrier. It is now known that permeability of the blood-brain barrier increases after major histocompatibility complex class I expression (Fabry et al, 1994), activated lymphocyte interaction, and change in neuronal cell adhesion molecules (71, 72). Based on review of literature and results reported here, we propose the following chain of events, as shown in Figure 4, that may explain possible mechanisms of injury in autism:

1. In the course of a lifetime, the body is exposed to infectious agents, which mimic neuron-specific antigens, such as EBV, CMV, HHV-6, HTLV-1, HTLV-2, streptococcus, Chlamydia pneumoniae or even milk and gluten peptides.
2. Pre-existing auto-reactive T-cells are generated by molecular mimicry as a result of contact with dietary proteins and viral, bacterial, and parasitic antigens, which have sequence homologies or matched motifs with autoantigens.
3. Bacterial enterotoxins, viral antigens, and metals, such as mercury and lead, may increase adhesion molecules on brain endothelial cells. Toxic chemicals may also increase leukocyte function-associated antigens on activated T-cells.
4. Pre-existing autoreactive T-cells may transmigrate across the blood-brain barrier and induce the activation of local antigen-presenting cells, such as microglia and astrocytes.
5. By reacting to μ , γ , and κ opioid receptors on both lymphocytes and nerve cells, dietary peptides such as casomorphins, gluteomorphins and others may change the level of cytokine production and interfere with neuroimmune communication (19, 20, 53; Figures 2, 3).
6. This neuroimmune miscommunication may result in production of IL-2, INF- γ and TNF- α by T-helper-I autoreactive cells and TNF- α by the antigen presenting cells (astrocytes and microglia may result in oligodendrocyte damage and demyelination).
7. As a result of this sequence of events, MBP, MAG, MOG, α , β -crystallin and other antigens are released from neurofilaments and enter the circulatory

system. This results in immune reactions, such as the formation of plasma cells with the capacity of producing IgG, IgM and IgA antibodies against neuron-specific antigens.

8. These antibodies may cross the blood-brain barrier and combine with brain tissue antigens to form immune complexes, thus causing further damage to the neurological tissue. The antibodies, along with toxic biological weaponry, such as arachidonic acid and free radicals, can "chew off" neuron myelin and impair electrical transmission between a muscle and the central nervous system.
9. This hypothesis may explain significant differences in levels of pathogenic autoantibodies between control subjects and patients exposed to toxic chemicals and metals (11, 15, 19, 20, 53).

[0074] An embodiment provides for a method for determining etiology of an autistic spectrum disorder in a patient, comprising the steps of:

- a) determining a level of at least one infectious agent derived antigen or antibody against an infectious agent derived antigen, at least one toxic chemical derived antigen or an antibody against a toxic chemical, and at least one dietary protein derived antigen or antibody against a dietary protein, in one or more samples from the patient;
- b) comparing the level of antigens and/or antibodies determined in step a) with a normal level of the antigens and/or antibodies from control subjects, wherein
 - (i) normal level or lower than normal level of antigens and/or antibodies for the each of said antigens indicate absence of an etiology of autistic spectrum disorder from presence of said antigens; and
 - (iv) higher than normal level of antigens and/or antibodies for one or more of said antigens and/or antibodies indicates a likelihood of the autistic spectrum disorder being based on the presence of said antigens.

[0075] Another embodiment provides for a method for determining etiology of an autistic spectrum disorder in a patient, comprising the steps of:

- a) determining a level of antibodies to a self-tissue or peptide in one or more samples from the patient; and
- b) comparing the level of antibodies determined in step a) with a normal level of the antibodies from control subjects, wherein
 - (i) normal level or lower than normal levels of antibodies indicate absence of etiology of autistic spectrum disorder from presence of said antibodies; and
 - (ii) higher than normal level of the antibodies indicates a likelihood of the autistic spectrum disorder being based on the presence of said antibodies.

[0076] Preferred self-tissue or peptide include, but is not limited to, tissue and cell antigens, receptors, mediators, enzymes, and neurotransmitters. More specifically, preferred self-tissue or peptide include, but is not limited to, digestive enzymes (pepsin, trypsin, chymotrypsin), aminopeptidase, dipeptidyl peptidase, CD26, DPPI IV, CD13, CD69, transglutaminase, epithelial cells, brush border antigens and enzymes, colon tissue antigens, gastrin, gastrin inhibitory polypeptide, secretin, motilin, enkephelin, substance P, somatostatin, and serotonin. When a preferred self-tissue antigen or peptide is a neurotransmitter or a neurotransmitter receptor, the preferred self-tissue antigen or peptide is selected from the group consisting of serotonin receptor, serotonin, somatostatin, vasoactive intestinal peptide, pro-dynorphin, dynorphin, dipeptidylpeptidase IV, and complex dipeptidylpeptidase IV.

Material And Methods

[0077] An immunoassay, such as ELISA, RAST, DotBlot, Western Blot and others can be used in embodiments.

[0078] The following antigens, proteins, peptides, enzymes, tissue receptors, lymphocyte receptors, neurotransmitters listed below are representative of antigens used in assays of preferred embodiments.

MBP Sequence 87-106	VVHFFKNIVTPRTPPPSQGK
MBP Sequence 83-89	ENPVVHFFKNIVTPRTP

MBP Sequence 1-11	ASQKRPSQRSK
MBP Sequence 200-211	ANMQRQAVPTL
Proteolipid Protein Sequence 40-60	TGTEKLIETYFSKNYQDYEYL
Proteolipid Protein Sequence 89-106	GFYTTGAVRQIFGDYKTT
Proteolipid Protein Sequence 103-120	YKTTICGKGLSATVTGGQ
Proteolipid Protein Sequence 125-143	SRGQHQAHSLERVCHCLGK
Proteolipid Protein Sequence 139-154	HCLGKWLGHDPDKFVGI
Transaldolase Protein Sequence 11-25	MESALDQLKQFTTVV
Transaldolase Protein Sequence 21-35	ETTVVADTGDFHAID
Transaldolase Protein Sequence 31-45	FHAIDEYKPQDATTN
Transaldolase Protein Sequence 71-85	KLGGSQEDQIKNAID
Transaldolase Protein Sequence 81-95	KNAIDKLFVLFGAEI
Transaldolase Protein Sequence 261-275	GELLQDNAKLVPVLS
Transaldolase Protein Sequence 271-285	VPVLSAKAAQASDLE
Transaldolase Protein Sequence 311-325	GIRKFAADAVKLERM
MOG Sequence 1-20	GQFRVIGPRHPIRALVGDEV
MOG Sequence 61-80	QAPEYRGRTELLKDAIGEGK
MOG Sequence 101-120	RDHSYQEEAAMELKVEDPFY
MOG Sequence 145-160	VFLCLQYRLRGKLRAE
MAG Sequence 37-60	REIVDRKYSICKSGCFYQKKEEDW
Sodium Ion Channel Na 1.2	TVTVPIALGESDFENLNTEEFSSSESDM
Na 1.3	TVTVPIAVGESDFENLNTEEFSSSESEL
Na 1.1	TVTVPIAVGESDFENLNTEDFSSSESDL
Na 1.6	TVRVPIAVGESDFENLNTEDEVSSSESDP
Glutamate Receptor	ANEYERFVPFSDQQISNDAAC
Cerebellar peptides	FLEDVPLLEDIPLLEDVPLLED
	FLEDVPLLEDIPLLEDVP
	LLEDTDFLEDPDFLEAID
Amyloid β	DAEFRHDSGYEVHHQKLVFFAEDVGSNKGAIIGLMVGGGVIA
CD69-Human	MECEKNLYWICNKPYK

Zinc Finger Protein	PYKCPECGKSFSQKSDLVKHQRTHTG
Glucose Regulated Protein-78 (GRP-78)	EEEDKKEDVGTVVGI
Vasoactive Intestinal Peptide	NYTRLRKQMAVKKYL
Gliadin Peptides	QFPRPQQPYPQPQPQYSQPQQ QPYPQPQPQYSQPQQPISQQQ QFLGQQQPFPQPQPYPQPQPF PLVQQQQFLGQQQPFPQPQPY HNVVHAILHQQQQQQQEQKQ NPSQQQPQEQVPLVQQQ QQLPQPQPQQSFPQQQPF
Gluteomorphin	YPFPGPIP
Casomorphin	GYPTYGGWL
Secretin (human)	HSDGTFTSELSRLREGARLQRLQGLV
Campylobacter Jejuni Toxin	TPPLLAAILMLASLRSHIVSDHFPVNFRKF
α -S1 Casein	RPKHPIKHQGLPQEVNLNENLLRFFVAPFPEVFGKEKVNELSKDIGSESTDEQAMEDIK QMEAESISSSEEIVPNSVEQKHIQKEDVPSEYLGYLEQLLRLKKYKVPQLEIVPNSAE ERLHSMKEGIHAQQKEPMIGVNQELAYFYPELFRQFYQLDAYPSGAWYYVPLGTQY TDAPSFSDIPNPIGSENSEKTTMPLW
α -S2 Casein	MKEGIHAQQK YQKFALPQYL
K Casein	KDERFFSDKI SPPEINTVQV
Vasoactive Intestinal Peptide	HSDAVFTDNYTRLRKQMAVKKYLNSILN
Somatostatin	YSANSNPAMAPRERKAGCKNFFWKFTFTSC
Substance P	RQKPQQFFGLM
Oxytocin	CYKQNCPLG
Pancreatic Peptide	APLEPVYPGDNATPEQMAQYAADLRRYINMLTRPRY
Gastrin-1	EGPWLEEEEEAYGWMDF
Big Gastrin-1	ELGPQGPPHLVADPSKKQGPWLEEEEEAYGWMDF

Gastrin Releasing Peptide	VPLPAGGGTVLTKMYPRGNHWAVGHLM
Enkephalin	YGGFLM
β -Endorphin	YGGFMTSEKSQTPLVTLFKNAIHKNAKKGE
Big Endorphin	CSCSSLMDKECVYFCHLDIIVVNTPEHVVPYGLGSPRS
Dynorphin A	YGGFLRRIRPKLKWDNQ
Dynorphin B	YGGFLRRQFKVVT
Serotonin Receptor	MPHLLSGFLEVTASPAPTWDAP IFGHFFCNVFIAMDVMCCTASI LKLAERPERSEFVLQNSDHCGK
Fibrillin	SFRPGSRGGSRG
Calreticullin	EQFLDGDGWTSRWIESGLQTSQ
Motillin	FVPIFTYGELQRMQEKERNKGQ
Chlamydia HSP-60	LKQIAAHAGKEGAIIFQQVM
Human HSP-60	1-20 MLRLPTVFRQMRPVSRLAP 16-35 RVLAPHLTRAYAKDVKFGAD 31-50 KFGADARALMLQGVDLLADA 46-65 LLADAVAVTMGPKGRTVIE 61-80 TVIEQSWGSPKVTKDGVTV 76-95 DGVTVAKSIDLKDKYKNIGA 91-110 KNIGAKLVQDVANNTNEEAG 106-125 NEEAGDGTTTATVLARSIK 121-140 RSIKEGFEEKISKGANPVEI 136-155 NPVEIRRGVMLAVDAVIAEL 151-170 VIAELKKQSKPVTTPPEIAQ 166-185 EEIAQVATISANGDKEIGNI 181-199 EIGNIISDAMKKVGRKGV 195-214 RKGVITVKDGKTLNDELEII 210-229 ELEIIEGMKFDRGYISPYFI 225-244 SPYFINTSKGQKCEFQDAYV 240-259 QDAYVLLSEKKISSIQSIVP

255-275	QSIVPALEIANAHRKPLVIA
271-290	LVIIAEDVDGEALSTLVLNR
286-305	LVLNRLKVGLQVVAVKAPGF
301-320	KAPGFGDNRKNQLKDMAIAT
316-335	MAIATGGAVFGEEGLTLNLE
331-350	TLNLEDVQPHDLGKVGEVIV
346-365	GEVIVTKDDAMLLKGKGDKA
361-380	KGDKAQIEKRIQEIEQLDV
376-395	EQLDVTTSEYEKEKLNERLA
391-410	NERLAKLSDGVAVLKVGGS
406-425	VGGTDVEVNEKKDRVTDAL
421-440	VDALNATRAAVEEGIVLGG
436-455	IVLGGGCALLRCIPALDSL
451-470	LDSLTPANEDQKIGIEIKR
466-485	EIKRTLKIPAMTIAKNAGV
481-500	KNAGVEGSLIVEKIMQSSSE
496-515	QSSSEVGYDAMAGDFVNMVE
511-530	VNMVEKGIIDPTKVVRTALL
526-545	RTALLDAAGVASLLTTAEVV
541-560	TAEVVVTEIPKEEKDPGMGA
556-573	PGMGAMGGMGGGMGGGMF
437-460	VLGGGVLLLRVIPALDSLTPANED

Dipeptidylpeptidase peptides

Peptide 1	MKTPWRVLLGLLGAAALVTIITVPVLLNK
Peptide 2	MAEYGNSSVFLENSTFDEFGH
Peptide 3	KRQLITEERIPNNTQWVTWSP
Peptide 4	NGTFLAYAQFNDTEVPLIEYS
Peptide 5	VTNATSIQITAPASMLIGDHY
Peptide 6	IQNYSVMDICDYDESSGRWNC
Peptide 7	NSFYKIISNEEGYRHICYFQI

Peptide 8	NVQMPSKKLDFILNETKFWY
Peptide 9	PEDNLDHYRNSTVMSRAENFK
Peptide 10	TAHQHIYTHMSHFQKCFSLP

Figure 5 shows sequences for Dipeptidyl Peptidase IV during intestinal differentiation which is also useful in assays of preferred embodiments. Figure 5 compares the amino acid sequences of human, rat, and mouse DPP IV, respectively. These sequences are aligned. The potential sites for phosphorylation (T or S) and for N-glycosylation (NXT) are displayed as underlined. Preferred embodiments include the sequences listed above, along with counterparts which have post-translational modifications.

ELISA Procedure

[0079] Enzyme-linked immunosorbent assay (ELISA) was used for testing antibodies against different neuron-specific antigens, milk and bacterial peptides in the sera of patients with autism and control subjects. Antigens or peptides proteins, enzymes, neurotransmitters were dissolved in methanol at a concentration of 1.0 mg/ml, then diluted 1:100 in 0.1 M carbonate-bicarbonate buffer, pH 9.5, and 50 μ l were added to each well of a polystyrene flat-bottom ELISA plate. Plates were incubated overnight at 4°C and then washed three times with 20 mM tris-buffered saline (TBS) containing 0.05% Tween 20, pH 7.4. The nonspecific binding of immunoglobulins was prevented by adding a mixture of 1.5% bovine serum albumin (BSA) and 1.5% gelatin in TBS, and then incubating for 2 h at room temperature, and then overnight at 4°C. Plates were washed as in the above, and then serum samples diluted 1:100 in 1% BSA-TBS were added to duplicate wells and incubated for 2 h at room temperature. Plates were washed, and then enzyme-conjugated or alkaline goat anti-human IgG, IgM or IgA antiserum (KPI, Gaithersburg, Maryland) were optimally diluted in 1% BSA-TBS was added to each well; the plate was incubated for an additional 2 h at room temperature. After washing five times with TBS-Tween buffer, the enzyme reaction was started by adding 100 μ l of substrate. After 45 min, the reaction was stopped with 50 μ l of stop solution. The optical density (O.D.) was read at 405 or 492 nm by means of a microtiter reader. Several control wells containing all reagents, but human serum, were used for detecting nonspecific binding.

[0080] Based on the above, a series of ELISA experiments was performed to establish the binding specificity of peptides, SK and mercury to CD26 and CD69. The plates were coated with CD26 or CD69 first and then with 1% BSA or HSA for inhibition of non-specific binding to microplate wells. Gliadin, casein peptides, SK and ethyl mercury were then added. Plates were incubated for one hour at 37°C and washed five times for removal of unbound competing antigens. Then, for demonstration of casein, gliadin, SK and mercury binding to CD26 and CD69 purified enzyme labeled rabbit anti-CD26 and CD69 were added to different wells. After proper incubation and washing, binding of these peptides and proteins to CD26 and CD69 was measured by addition of peroxidase substrate and measurement of color development at 492 nm. Binding of dietary peptides, SK and ethyl mercury to CD26 and CD69 was demonstrated by % inhibition in binding of CD26 or CD69 to anti-CD26 and anti-CD69 to its specific antibody and different peptides, SK or mercury was calculated by using the following formula:

$$\begin{array}{l} \text{\% binding of gliadin to} \\ \text{CD26 and inhibition of} \\ \text{anti-CD26 binding to CD26} \end{array} = 100 - \frac{\text{O.D. after addition of peptide} - \text{background}}{\text{O.D. of CD 26 + antiCD 26} - \text{background}} \frac{\text{O.D.}}{\text{O.D.}} (100)$$

Example:

O.D. for CD26 + anti-CD26 = 2.16

O.D. for CD26 + gliadin and anti-CD26 = 1.19

O.D. for background = 0.28

% binding of gliadin to CD26 = 100 —

$$\frac{1.19 - 0.28}{2.16 - 0.28} (100) = 100 - \frac{0.91}{1.88} (100) = 100 - 48 = 52$$

Bacterial Toxins Ethyl Mercury And Dietary Peptides Bind To Dipeptidylpeptidase IV (CD26) And CD69 And Induce Antibody Production

[0081] For an examination of the possible involvement of gliadin, casein peptides, SK and ethyl mercury in the production of autoantibodies against CD26 and CD69, calculations of the simultaneous elevation of these antibodies in patients' sera were made, as is summarized in Table 1. Analysis of data showed that while some patients had elevated IgG, IgM or IgA against 1 or 2 of 6 tested antigens, different subgroups showed simultaneous

elevation in IgG, IgM or IgA antibodies not only against CD26 and CD69, but also against gliadin (GLI), casein (CA), peptides, SK, ethyl mercury (Hg) or a combination of CD26 or CD69 + GLI + CA, or GLI + CA + SK + Hg (Table 1). For example, patients #2, 4, 13, 19, 23, 24, 29, 30, 31, 32, 34 and 38 demonstrated IgG antibody elevation not only against CD69 but also against gliadin, casein, SK or their combinations. Similarly, patients #4, 13, 28, 32 and 48 demonstrated IgM antibody elevation against CD26 or CD69 in combinations with gliadin, casein and SK; patients #1, 2, 3, 4, 6, 13, 14, 16, 21, 24, 29, 33, 41 and 42 demonstrated IgA antibodies against CD26, CD69, gliadin, casein or SK. In patient #13, with the exclusion of IgG and IgA against mercury, all other measurements were highly elevated. This suggests that the patient not only reacted against CD26 and CD69, but also against gliadin, casein, SK, and mercury as well (Table 1).

[0082] This simultaneous elevation of anti-CD26, casein, gliadin, SK, and mercury in children with autism further supports the argument for the binding of these dietary and bacterial peptides or antigens to tissue enzymes (dipeptidylpeptidase) or lymphocyte receptors (CD26). The possible mechanism of action for DPP IV binding with dietary peptides, infections, and xenobiotics, resulting in antibody production against DPP IV, gliadin, casein, SK and mercury is shown in Fig. 6.

Table I - Simultaneous Detection of Antibodies Against DC26, DC69, Gliadin (Gli), Casein Peptides (CA), SK, and Ethyl Mercury (Hg) in Children with Autism

Specimen #	IgG						IgM						IgA					
	CD26	CD69	Gli	CA	SK	Hg	CD26	CD69	Gli	CA	SK	Hg	CD26	CD69	Gli	CA	SK	Hg
1	-	-	M	M	M	M	-	+	-	-	+	-	M	M	M	M	M	-
2	M	M	M	M	M	-	-	-	-	-	+	-	M	M	M	M	M	+
3	-	-	-	-	+	-	+	+	-	+	+	+	M	M	M	-	+	-
4	-	M	M	M	M	-	-	M	M	M	M	-	+	M	M	M	M	-
5	-	-	-	-	-	-	-	-	-	-	+	+	-	-	-	-	-	-
6	-	-	-	-	-	-	+	+	-	-	+	-	M	M	M	M	-	-
7	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-
8	+	-	+	-	-	-	-	-	-	-	-	-	-	-	+	+	-	-
9	-	-	-	+	-	-	-	+	-	-	-	-	-	-	-	-	-	-
10	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-
11	-	+	-	-	-	-	+	-	-	-	-	-	-	-	-	-	-	-
12	-	-	-	-	-	-	+	-	-	-	+	+	-	-	-	-	+	-
13	M	M	M	M	M	-	M	M	M	M	M	+	M	M	M	M	M	-
14	-	-	-	-	-	-	-	-	+	+	+	-	M	M	M	M	-	-
15	+	-	-	+	+	-	-	-	+	+	+	-	-	-	-	-	-	-
16	M	-	M	M	M	-	+	-	-	-	+	-	M	M	M	M	-	-
17	-	-	-	-	-	-	-	-	+	+	-	-	-	-	-	-	-	-
18	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-
19	M	M	M	M	M	M	-	-	-	-	+	-	+	-	+	+	-	-
20	-	-	-	+	+	-	+	-	+	-	+	-	-	-	-	-	-	-
21	+	-	-	-	-	+	+	-	-	+	-	-	M	M	M	-	M	-
22	-	-	-	-	-	-	-	-	+	-	-	-	-	+	-	+	-	-
23	M	M	M	M	M	M	-	-	-	-	+	+	-	-	-	+	-	-
24	+	+	+	-	-	-	-	-	-	-	+	-	+	+	+	-	-	-
25	M	-	M	M	M	M	-	-	-	-	-	-	-	-	-	-	-	-
26	-	+	-	+	-	-	+	+	-	-	+	+	-	-	-	-	-	+
27	+	+	+	-	-	+	+	-	-	-	+	-	-	-	-	-	-	-
28	-	-	-	-	-	+	-	M	M	M	M	-	+	+	-	-	-	-
29	M	M	M	M	-	+	-	-	+	+	+	+	M	M	M	M	-	-
30	+	+	+	-	-	-	-	-	+	+	-	-	-	-	+	+	-	-
31	M	M	M	M	-	-	+	+	-	-	-	-	+	+	-	-	-	-
32	M	M	M	M	M	-	M	-	M	M	M	M	-	-	+	+	+	-
33	+	-	-	-	-	-	+	-	-	+	+	-	+	+	+	-	-	-
34	M	M	M	M	-	+	-	-	+	+	+	+	-	+	+	+	-	+
35	-	-	-	-	-	+	-	-	-	-	+	+	-	+	-	-	+	+
36	-	+	-	-	-	-	+	-	-	-	-	-	+	+	-	-	-	-
37	-	-	-	-	-	-	-	-	+	+	-	-	-	-	+	+	-	-
38	M	M	M	M	-	-	+	-	-	-	-	-	+	+	-	-	-	-
39	-	-	-	-	+	-	-	-	+	+	-	+	-	-	-	-	+	-
40	+	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-
41	-	-	+	+	-	-	+	-	-	-	-	-	M	M	M	M	-	-
42	+	-	+	-	+	+	+	-	+	+	-	+	M	M	M	M	M	+
43	-	+	-	-	-	+	-	-	-	-	-	+	-	+	-	-	-	-
44	+	-	-	+	-	-	-	-	+	+	-	-	+	+	-	-	-	-
45	+	-	-	-	+	+	+	+	-	-	-	-	-	-	+	+	+	-
46	-	-	-	-	-	-	-	-	+	+	+	-	+	-	-	-	-	-
47	+	-	+	+	-	-	+	+	-	-	-	-	+	+	-	-	-	-
48	-	-	+	-	-	+	M	M	M	-	M	M	-	-	+	+	-	-
49	-	+	+	-	-	-	-	-	+	-	+	+	-	-	+	+	-	-
50	+	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-

- = negative

+ = positive

M = multiple positive results

Binding of Gliadin, Casein Peptides, SK and Ethyl Mercury to CD26 and CD69

[0083] For demonstration of gliadin, casein, SK and mercury binding to CD26 and CD69, polyclonal antibodies raised against CD26 or CD69 and labeled with enzyme were added to different wells coated either with CD26 or CD69. Rabbit anti-CD26 reacted only with wells coated with CD26 that resulted in an ELISA O.D. of 2.16. Adding rabbit anti-CD69 to wells coated with CD69 gave an ELISA O.D. of 1.92. Adding gliadin, casein, SK and mercury to wells coated with either CD69 or CD26 caused 52%, 44%, 77% and 73% inhibition in binding of anti-CD26 to CD26. Similarly, in wells coated with CD69, gliadin, casein, SK and mercury caused 57%, 28%, 86% and 81% inhibition in Anti-CD69 to CD69 coated wells. This reduction in ELISA O.D.s of anti-CD26 or anti-CD69 binding to CD26 or CD69, is an indication of gliadin, casein, SK and mercury binding to CD26 and CD69 (Table 2).

TABLE 2: Inhibition of anti-CD26 and anti CD-69 by gliadin, casein, streptokinase and ethyl mercury which reflects the binding of these molecules to CD26 and CD69 coated plates.

BG = background

Microwell Coated With:	Peroxidase Labeled Rabbit Anti:	ELISA O.D. at 492 nm	% Binding of Gliadin, Casein, Streptokinase, or Mercury to CD26 and CD69
BSA + HSA	CD26	0.28	B.G.
BSA + HSA	CD69	0.24	B.G.
BSA + HSA	Gliadin	0.31	B.G.
BSA + HSA	Casein	0.29	B.G.
BSA + HSA	SK	0.33	B.G.
CD26 + BSA + HSA	CD26	2.16	-
CD26 + Gliadin	CD26	1.19	52
CD26 + Casein	CD26	1.34	44
CD26 + SK	CD26	0.72	77
CD26 + Ethyl Mercury	CD26	0.79	73
CD69 + BSA + HSA	CD69	1.92	-
CD69 + Gliadin	CD69	0.97	57
CD69 + Casein	CD69	1.45	28
CD69 + SK	CD69	0.48	86
CD69 + Ethyl Mercury	CD69	0.56	81

Binding of SK Gliadin and HSP-60 to DPP IV and Other Enzymes or Receptors

[0084] In additional experiments, we showed that bacterial toxins and heat shock proteins could promote development of peptidase antibodies in children with autism and patients with autoimmune disease. In these experiments, by searching for a mechanism underlying autoimmunity in autism, we postulated that gliadin peptides, heat shock protein (HSP-60) and streptokinase (SK) bind to different peptidases. Binding results in autoimmunity. We assessed this hypothesis in patients with autism and in those with mixed connective tissue diseases. Concomitant with the appearance of anti-gliadin and anti-HSP antibodies, children with autism, and patients with autoimmune disease developed anti-DPPI, anti-DPP IV and anti-CD 13 autoantibodies. These antibodies may be synthesized as a result of gliadin and HSP-60 binding to different peptidases since a significant percentage of autoimmune and autistic sera were associated with elevated IgG, IgM or IgA antibodies against all three peptidases, gliadin and HSP-60. These antibodies are specific since immune absorption demonstrated that only specific antigens (i.e., DPP IV absorption of anti-DPP IV significantly reduced IgG, IgM and IgA antibody levels). For direct demonstration of SK, HSP-60 and gliadin peptides binding to DPP IV, microtiter wells were coated with DPP IV and with SK, HSP-60 and gliadin. Finally they were reacted with rabbit anti-DPP IV, or anti-SK, anti-HSP-60 and anti-gliadin. Addition of SK, HSP-60 and gliadin peptides to DPP IV resulted in 27-43% inhibition of DPP IV anti-DPP IV reaction. Furthermore, addition of anti-SK, anti-HSP-60 and anti-gliadin to DPP IV + peptides caused 18-20% enhancement of antigen-antibody reaction. These results further support: binding of SK, gliadin and HSP to DPP IV. We propose that superantigens (e.g., SK, HSP-60), dietary proteins (e.g., gliadin peptides) in individuals with predisposing HLA molecules bind to aminopeptidases and induce autoantibodies against peptides and tissue antigens. From our results we conclude that binding of bacterial superantigens to DPP IV, DPP I or CD13 can be responsible for autoantibody production in children with autism and in patients with autoimmune diseases (12, 14, 15).

Table 3 – Percent Elevation of Antibodies Against DPP IV, DPPI, CD13, Gliadin Peptidase and HSP-60 in Controls and Patients with Autism and Autoimmune Disease

ANTIGENS	% IgG Elevation in:				% IgM Elevation in:				% IgA Elevation in:			
	Children Control	Autism	Adults Control	Auto-immune	Children Control	Autism	Adults Control	Auto-immune	Children Control	Autism	Adults Control	Auto-immune
DPP IV	10	54	14	64	8	50	8	46	6	44	4	58
DPP I	8	56	14	60	10	46	16	54	10	46	14	56
CD13	8	40	6	28	6	18	6	18	12	48	8	50
Gliadin Peptide	12	42	18	62	8	50	20	48	6	44	18	52
HSP-60	16	36	22	52	8	44	18	48	14	50	14	42

Table 4 – Multiple Comparisons and Means for Groups in Homogenous Subsets with Scheffé's Post Hoc Tests

	<u>SUBSET</u>				<u>SUBSET</u>			<u>SUBSET</u>	
	1	2	3		1	2	3	1	2
A. DPP IV IgG in: Control-Children Control-Adults Autism Autoimmune	.1432 .1994	.3488 .4282		A. DPP IV IgM in: Control-Children Control-Adults Autism Autoimmune	.1694 .1826	.3260 .3700		A. DPP IV IgA in: Control-Children Control-Adults Autism Autoimmune	.1336 .1654 .3620 .5120
B. DPP I IgG in: Control-Children Control-Adults Autism Autoimmune	.1446 .1492	.3580 .3940		B. DPP I IgM in: Control-Children Control-Adults Autism Autoimmune	.1492 .1480	.2980	.4300	B. DPP I IgA in: Control-Children Control-Adults Autism Autoimmune	.1508 .1546 .3320 .4560
C. CD13 IgG in: Control-Children Control-Adults Autism Autoimmune	.1432 .1994	.2900 .2460		C. CD13 IgM in: Control-Children Control-Adults Autism Autoimmune	.1310 .1400 .1700 .1680			C. CD13 IgA in: Control-Children Control-Adults Autism Autoimmune	.1484 .1218 .4040 .3900
D. Gliadin Peptide IgG in: Control-Children Control-Adults Autism Autoimmune	.1640 .2140	.2120 .3600	.3600 .5060	D. Gliadin Peptide IgM in: Control-Children Control-Adults Autism Autoimmune	.1460 .1920	.3580 .4640		D. Gliadin Peptide IgA in: Control-Children Control-Adults Autism Autoimmune	.1454 .1860 .3680 .4520
E. HSP-60 Peptide IgG in: Control-Children Control-Adults Autism Autoimmune	.1620 .1980 .2800	.5020		E. HSP-60 Peptide IgM in: Control-Children Control-Adults Autism Autoimmune	.1300 .1770	.1770 .3380	.3380 .4880	E. HSP-60 Peptide IgA in: Control-Children Control-Adults Autism Autoimmune	.1618 .1636 .6100 .5820

NOTES: Means for groups in homogenous subsets are displayed based on type III sum of squares (sample size of 200, with 50 subjects in each group). Means that are reported in the same subset are statistically similar. For example, means for IgG (DPP I) for the control-children and control-adults groups are .1446 and .1492, respectively, which are statistically alike. Similarly, the means for the autism (.3580) and autoimmune groups (.3940) are statistically the same. However, the means for control-children are significantly different from the autism or autoimmune group. Similarly, the means for the control-adults are statistically different from both the autism and autoimmune groups. Also note that the control groups (.1492 & .1480) are similar for IgM (DPP I), but autism (.2980) and autoimmune (.4300) are not statistically alike. Potentially, up to four subsets could be formed, simply because for each dependent variable we have four experimental groups. When this is the case each mean's group should be reported in a separate subset. However, if all four means are statistically alike, all should be reported in one subset. For example, for CD13 IgM, all means are reported in the sample subset, indicating that no difference between the four groups was detected.

[0085] Table 5 shows percent elevation of IgG, IgM, and IgA antibody levels against digestive enzymes, tissue enzymes, lymphocyte receptors, neuroimmune communicators, gliadin peptides, casein peptides, bacterial antigens, bacterial toxins, and ethyl mercury in controls and children with autism at a cut-off of 2 standard deviations above the mean of controls.

Table 5: Percent Elevation of IgG, IgM, and IgA Antibody Levels Against Digestive Enzymes, Tissue Enzymes, Lymphocyte Receptors, Neuroimmune Communicators, Gliadin Peptides, Casein Peptides, Bacterial Antigens, Bacterial Toxins, and Ethyl Mercury in Controls and Children with Autism at a Cut-Off of 2 Standard Deviations above the Mean of Controls

Antigen	IgG		IgM		IgA	
	Controls	Patients	Controls	Patients	Controls	Patients
DPP IV	14	54	10	50	8	44
DPPI	14	56	12	46	10	46
CD13	8	40	8	18	10	48
CD69	6	36	4	28	4	46
Pepsin	4	38	2	32	4	42
Trypsin	6	44	4	36	6	48
Chymotrypsin	8	48	6	38	8	46
Secretin	12	52	8	40	10	50
Transglutaminase	10	44	6	42	8	42
Gastrin	12	46	8	34	6	38
Motilin	14	52	12	48	12	46
Vasoactive Intestinal peptide	8	48	6	32	10	52
Oxytocin	6	32	4	24	6	32
Glucose regulated protein	4	28	2	18	2	24
Tropomyosin	8	36	4	26	4	32

Gliadin peptides	8	42	8	50	10	44
Casein peptides	10	42	8	34	8	42
Heat shock protein	6	36	8	44	10	50
Streptokinase	2	18	4	48	2	24
Ethyl Mercury	4	28	2	30	0	10
Serotonin	6	30	8	36	12	46
Enkephalin	5	36	6	42	3	21
MBP	15	58	18	73	14	5
PLP	7	46	5	39	4	37
Transaldolase	4	29	2	21	1	19
MOG	11	48	19	63	9	40
MAG	8	49	11	62	4	27
Sodium channel	6	38	5	33	3	28
Glutamate receptor	4	42	6	39	2	31
Cerebellar	5	49	3	41	4	35
Amyloid β	1	23	2	20	1	15
Tubulin	8	49	10	63	10	42
Neurofilaments	4	48	8	58	9	38
Zinc finger protein	1	34	2	27	1	22
Somatostatin	3	39	2	34	1	26
C-jejuni	4	44	3	38	2	27
Pancreatic polypeptide	3	27	2	25	0	15
B-Endorphin	5	36	3	32	0	18
Endothelin	2	35	1	27	1	0
Dynorphins	2	37	1	30	0	16
Serotonin receptor	3	36	3	28	1	22
Fibrillarin	2	44	1	40	1	27
Calreticullin	1	30	0	24	0	15

Antibodies Against Neuron-Specific Antigens In Autism

[0086] Until recently, there has been little direct evidence readily available in support of the molecular mimicry hypothesis and to clearly delineate the role of infectious agents as a cause for neurological disorders. For example, studies in mice have shown that infection with Theiler's virus elicits an inflammatory response in the CNS that progresses to chronic experimental autoimmune encephalomyelitis (28). Epitopes of Streptococcal M proteins have also been shown to evoke antibodies that cross-react with human brain neuronal cell basal ganglia, which are potentially involved in the pathogenesis of Sydenham's chorea (associated with acute rheumatic fever). Very recently, a rather elaborate experiment of a well-characterized rat model of MS has been used to investigate the casual relationship between infections and MS. Investigators identified a 20-mer peptide from a protein specific to *C. pneumonia*, which shares a 7-amino acid motif with a critical epitope of myelin basic protein, a major CNS antigen targeted by the autoimmune response in MS. This bacterial peptide induces a Th1 response, accompanied by severe clinical and histological experimental autoimmune encephalomyelitis in Lewis rats, a condition closely reflective of many aspects of MS. Studies with peptide analogues suggest that different populations of encephalitogenic T cells are activated by the *C. pneumoniae* and myelin basic protein antigens (29, 30). Based on these findings and research, we hypothesized that if infectious antigens or toxic chemicals cause the blood-brain barrier to become more permeable, then antibodies (such as IgG, IgM, and IgA) to neurologic antigens or pathogenic peptides should be detectable in the blood of patients with autism.

[0087] To examine this hypothesis, we used different purified protein and synthetic peptides in a highly specific, in-house ELISA procedure with a low background of smaller than 0.1 O.D. at 492. Another advantage of this assay is the use of a second antibody, such as antihuman IgG, IgM or IgA, for identification of antibody isotypes. In many autoimmune diseases (including autoimmune neurological disorders), the isotypes IgM and IgA autoantibodies are considered to be more pathogenic than the IgG isotype. As shown in Table 6, it was found that all three isotype antibodies, whether alone or in combination, exhibited higher levels in autistic patients than in healthy control subjects. The purified

protein and synthetic peptides include myelin basic protein (MGP), myelin associated glycoprotein (MGP), ganglioside GM₁ (GM₁), sulfatide (SULF), chondroitin sulfate (CONSO₄), myelin oligodendrocytes glycoprotein (MOG), α , β -crystallin (α , β -CRYS), neuron-axon filament protein (NFB), glial fibrillary acidic protein (GFAP), tubulin, cerebellar purkinje cells (Cerebellar), glutamate receptor, ion channel, and transaldolase.

Table 6: Approximate Percent Elevation of IgG, IgM, and IgA Antibody Levels Against 13 Different Neural Antigens in Controls and Children with Autism at a Cut-Off of 2 standard deviations above the Mean of Controls

Antigen	IgG		IgM		IgA	
	Controls	Patients	Controls	Patients	Controls	Patients
MBP	14	57	16	61	12	48
MAG	9	49	12	56	5	29
GM ₁	6	52	9	59	3	37
SULF	4	51	13	64	6	41
CONSO ₄	7	46	12	60	5	42
MOG	11	43	11	58	7	39
α , β -CRYS	4	53	9	56	3	21
NF β and GFAP	6	49	8	53	6	27
Tubulin	12	50	10	56	8	36
Cerebellar	5	41	7	46	4	26
Glutamate receptor	3	37	5	39	2	23
Ion channel	1	19	2	17	1	14
Transaldolase	4	33	3	29	2	26

[0088] This simultaneous elevation of IgG, IgM and IgA antibodies against multiple neurological antigens indicates that an alteration of the blood-brain barrier by infectious agent antigens promotes the access of immunocompetent cells to many different nervous system antigens. Thus, immune cell reaction to the nervous system antigens is not limited only to neuronal and glial filament, but also against many other nervous system

antigens. See Vojdani et al., 2002, in the *Journal of Neuroimmunology*, vol. 129, pages 168-177, hereby incorporated by reference.

Simultaneous Detection of Antibody Against Gliadin And Cerebellar Peptides

[0089] By studying amino acid sequences of α -gliadin several peptides in particular a 33 mer was discovered to be responsible for cellular and humoral immune reactions in celiac disease. These gliadin peptides share between 20-30% similarity with cerebellar Purkinje cell antigens. Therefore, we developed peptide based ELISA assays for measuring antibodies against gliadin and cerebellar peptides simultaneously in children with autism.

[0090] Sera from 50 patients with autism were measured for simultaneous presence of IgG, IgM and IgA antibodies against gliadin and cerebellar peptides and compared to healthy controls. Results summarized showed that at 2 S.D. above the mean of controls, while 21 or 42% of patients with autism had elevated antibody levels against gliadin peptides, only 6 or 12% of control subjects had elevated antibodies against all these peptides. In comparison 18 or 36% of patients and 4 or 8% of controls demonstrated significantly elevated antibodies against cerebellar peptides. 17 of 21 subjects (80%) of patients with autism had simultaneous elevation in anti-gliadin and anti-cerebellar peptides, indicating cross-reaction between gliadin and cerebellar antigen, which results in these antibodies in a majority of gliadin reactive patients with autism.

[0091] Based on this antigenic similarity between milk butyrophilin, casein and gliadin peptides with myelin basic protein, myelin oligodendrocyte glycoprotein and cerebellar Purkinje cells, a casein and gliadin-free diet may be recommended for individuals with elevated milk and gliadin IgG, IgM or IgA antibodies.

[0092] In summary, we learn that autoantibodies to different tissue antigens in autism are produced by two different mechanisms of action: 1) by direct binding of infectious agent antigens or peptides, dietary proteins or peptides, or by binding of xenobiotics or their metabolites to tissue enzymes or cell receptors, inducing antibody production against the tissue antigens as well as bacterial, dietary or xenobiotics; and 2) many infectious agents, dietary proteins, and peptides share similar epitopes with different tissue antigens. Therefore,

immune responses against the infectious agents or dietary proteins result in autoimmune reactions with different tissue antigens, including brain cells. Based on these findings, we postulate that dietary and infectious antigens as well as xenobiotics play a role in the pathophysiology of autism. It is likely that environmental factors, including infection-induced injury, cause the release of neuronal antigens, which, through activation of inflammatory cells, could lead to autoimmune reactions in genetically susceptible individuals.

[0093] Since there is no single medical or laboratory marker that could be used for the diagnosis or follow-up treatment of children with autism, a protocol of testing for autistic spectrum disorders can further comprise: 1) assessment of immune function and imbalance in T-helper-1/T-helper-2 cytokines; 2) gut integrity or intestinal barrier function tests; 3) protection by metallothionein; and 4) assessment of serotonin level.

Assessment of Immune Function

[0094] These immune assays are recommended since strong lines of evidence suggest that the immune system plays an important role in the development of autism. Immune abnormalities in autism include changes in the numbers and activities of macrophages, T-cells, B-cells, and natural killer cells. Furthermore, a shift occurs from T-helper-1 to T-helper-2 T-cell type in autism as evidenced by a decrease in the production of Interleukin-2 (IL-2) and interferon- γ (IFN- γ) and an increased production on Interleukin-4 (IL-4). In other abnormalities in immune function, cytokine production and immunoglobulin levels may justify the use of intravenous immunoglobulin (IVIG) treatment or the use of biological response modifiers for regulation of the immune system. Tests for immune function include, but are not limited to, assessment of the following: Lymphocyte Subpopulation Analysis, Lymphocyte Immune Function Test, Natural Killer Cell Cytotoxic Activity, Immunoglobulins, T-Helper 1/T, C3 Complement, and C4 Complement.

Gut Integrity or Intestinal Barrier Function Tests

[0095] The intestinal barrier function test was developed since mucosal barrier dysfunction may result in gastrointestinal, cardiovascular, systemic immunity and autoimmunity.

[0096] Human beings harbor an incredibly complex and abundant ensemble of microbes. These resident bacteria shape our physiology in many ways. To investigate the importance of commensal bacteria in gastrointestinal health, germ-free mice were colonized with bacteroides and intestinal transcriptional responses were measured using DNA microarrays. Colonized bacteria modulated expression of genes involved in important intestinal function including:

1. Nutrient absorption
2. Lipid absorption capacity
3. Mucosal barrier fortification
4. Xenobiotic metabolism
5. Angiogenesis
6. Postnatal intestinal maturation

[0097] This shows the importance of host-microbial relationships in the GI tract and how gut bacteria and their products play a role in the induction and expression of normal immune responses, suggesting that changes in this flora may mediate abnormalities of system immunity.

[0098] In addition to measurement of antibodies against dietary proteins, yeast, aerobic and anaerobic bacteria, antibodies against secretin are measured. Similar to DPP IV, secretin is involved not only in digestion of peptides but also in neuroimmune communication. Therefore, demonstration of antibodies against secretin or DPP IV may justify enzyme replacement in children with autism.

[0099] The intestinal barrier function test was developed since, in our experience, microflora imbalance, intestinal barrier dysfunction, humoral immune deficiency, food allergy and autoimmunity cannot be fully understood in their diagnostic and therapeutic implications without coordination of all the components of the intestinal flora (yeast, aerobic bacteria, anaerobic bacteria, and dietary antigens).

[0100] Many of these conditions that adversely affect the intestinal flora have been mistakenly called “The Yeast Problem” and are tested by stool culture. While stool culture is a very powerful technique for detection of pathogenic bacteria, it may not detect any gastrointestinal microflora imbalance, since these organisms tend to bind to receptor sites on epithelial cells and secrete endotoxins, which damage local and distant tissues.

[0101] This systemic translocation of enteric bacteria and yeast plays a major role in the development of abnormal systemic immunity, which may result in multiple organ failure. As mentioned before, excessive uptake of bacterial, fungal, viral and food antigens into the circulation may induce immune response first in the form of IgM and, thereafter, in the form of IgG and IgA antibodies, which results in clinical conditions. For this reason, measurement of circulating IgM, IgG and IgA antibodies against specific antigens of intestinal bacterial and fungal flora is of considerable importance in the pathogenesis of immunologically mediated diseases including food allergies and autoimmunities. Again, this is the basis of our “Intestinal Barrier Function Test”.

[0102] In this test, we utilize a highly sensitive and accurate ELISA test method that measures the serum IgG, IgM and IgA specific antibody titers to the purified antigens from five different dietary proteins, three aerobic, two anaerobic microbes and a mixture of three different *Candida* species (*Candida albicans*, *Candida tropicalis* and *Candida krusei*).

[0103] Such quantitative and comparative test results may allow the determination of primary clinical conditions such as:

- Food allergy
- Intestinal Imbalance
- Gut barrier dysfunction
- Bacterial translocation
- Immunodeficiencies
- Candidiasis
- Autoimmunities

[0104] Intestinal barrier dysfunction may lead to polarized immune function, which may result in food allergy and intolerance. The intestinal immune system is characterized by a distinct profile of cells, adhesion molecules, cytokines and chemokines. In

addition, it has a predisposition to the induction of tolerance and bias towards productive or protective immunity that are dominated by production of IgA antibodies against food and commensal antigens. However, it is not clear why intestinal microenvironment results in polarized immune function.

[0105] It is believed that food proteins and antigens of commensal bacteria are taken up by immunoregulatory dendritic cells (DCs). In the absence of inflammation, prostaglandin E₂ (PGE₂) is produced by mesenchymal cells and macrophages. Transforming growth factor- β (TGF- β) and IL-10 is produced by epithelial cells, resulting in the maturation of DCs in the Peyer's patch or *Lamina propria*. These food and bacterial antigens are then presented to the naive CD4⁺ T-cells in mesenteric lymph nodes or Peyer's patch. These T-cells differentiate into regulatory T-cells, which produce interferon- γ and IL-10 or differentiate into T-helper-3 cells, which produce TGF- β . The immunological consequences are local IgA production, local immune homeostasis and systemic tolerance. However, when the body encounters pathogens, xenobiotics or some dietary peptides in the presence of inflammation, mesenchymal cells and macrophages not only fail to produce PGE₂ but they also express toll-like receptors. As a result, DCs in the Peyer's patch or *Lamina propria* become completely mature, taking up the antigen(s) and producing significant amounts of IgG, IgM and IgA antibodies against milk protein, milk peptides (casomorphin), wheat, corn and soy proteins, wheat peptides (gliadin peptides), gluteomorphins, and tissue enzymes (transglutaminase, DPP IV, aminopeptidases) (Figures 7, 8). Significant elevations in IgG, IgM or IgA antibodies against dietary proteins or peptides and their target tissue antigens (brush border enzymes) may justify treatment with the elimination diet and enzyme replacement or both. The Intestinal Barrier Function Test, disclosed in United States Patent #6,103,480, herein incorporated by reference, can include assessment of the following: Dietary Proteins (IgG, IgM, IgA), Yeast (IgG, IgM, IgA), Aerobic Bacteria (IgG, IgM, IgA), Anaerobic Bacteria (IgG, IgM, IgA), and Secretin (IgG, IgM, IgA).

Functional Metallothioneins Assay

[0106] Metallothioneins (MTs) have a major role to play in metal metabolism, and may also protect DNA against oxidative damage. MT protein has been found localized in the nucleus during S-phase.

[0107] MTs are a family of low-molecular-mass metal-binding protein isoforms. Although considered mainly cytoplasmic, MT has been found localized in the nucleus of the cell under different physiological conditions. For example, during the G₁-to-S-phase transition of the cell cycle or following metal toxicity, MT is found specifically in the nucleus. The function of MT in the nucleus is in protecting against metal toxicity and the harmful effects of oxidative stress, DNA damage and apoptosis induced by external stress.

[0108] Therefore, only the cellular activity of metallothionein is important for the assessment of metal-induced toxicity. The binding of metals to metallothionein may also result in autoantibody production against metallothionein in the nucleus.

[0109] Based on this information, the following panel were developed for determining etiology, and management of autism:

[0110] Since there is no single medical or laboratory marker that could be used for the diagnosis or follow-up treatment of children with autism. Therefore, one embodiment comprises a protocol of testing for autistic spectrum disorders can include a panel of tests chosen from the categories outlined in the next section, including Autism Panel – Short, Neuroimmunology of Autism Panel, Comprehensive Panel of all tests performed for autism.

AUTISM PANEL - SHORT

- Streptococcal Antigens (M5+, M12+, M19) (IgG, IgM, IgA)
- Measles Antibodies (IgG, IgM, IgA)
- HHV-6 (IgG, IgM, IgA)
- Gliadin Peptides Antibodies (IgG, IgM, IgA)
- Casein Peptides Antibodies (IgG, IgM, IgA)
- Fibrillarin (IgG, IgM, IgA)
- Dipeptidylpeptidase (DPP IV) Antibodies (IgG, IgM, IgA)
- Myelin Basic Protein Antibodies (IgG, IgM, IgA)
- Neurofilament Antibodies (IgG, IgM, IgA)

NEUROIMMUNOLOGY OF AUTISM PANEL

Antibodies to Dietary Proteins & Peptides (Food Allergy & Autoimmunity)

- Corn (IgG, IgM, IgA)
- Milk (IgG, IgM, IgA)
- Soy (IgG, IgM, IgA)
- Wheat Gluten/Gliadin (IgG, IgM, IgA)
- Casomorphin (IgG, IgM, IgA)
- Gluteomorphin (IgG, IgM, IgA)
- Secretin (IgG, IgM, IgA)
- Prodynorphin + Dynorphin (IgG, IgM, IgA)

Antibodies to Infectious Agents

- Clostridium Neurotoxin (IgG, IgM, IgA)
- Herpes Type 6 (IgG, IgM, IgA)
- Rubeola (Measles) (IgG, IgM, IgA)

Neurotoxicity and Autoimmune Reaction to Neuronal Cell Antigens

- Myelin Basic Protein (IgG, IgM, IgA)
- Neurofilament (IgG, IgM, IgA)

Neurotransmitters and Signal Transduction

- Serotonin Antibodies (IgG, IgM, IgA)
- Serotonin Receptor Antibodies (IgG, IgM, IgA)
- Somatostatin Antibodies (IgG, IgM, IgA)
- Dipeptidylpeptidase IV (CD26) (IgG, IgM, IgA)

COMPREHENSIVE PANEL OF ALL OF THE FOLLOWING TEST GROUPS:

FOOD ALLERGY AND INTOLERANCE

Antibody Testing on Blood

- Milk (IgG, IgM, IgA)
- Casomorphin (IgG, IgM, IgA)
- Wheat Gluten/Gliadin (IgG, IgM, IgA)

- Gluteomorphin (IgG, IgM, IgA)
- Transglutaminase (IgG, IgM, IgA)
- Corn (IgG, IgM, IgA)
- Soy (IgG, IgM, IgA)

INFECTIOUS AGENTS AND RESPONSE TO VACCINATIONS

- Measles (Rubeola) (IgG, IgM, IgA)
- Mumps (IgG, IgM, IgA)
- Rubella (IgG, IgM, IgA)
- Diphtheria Toxoid (IgG, IgM, IgA)
- Pertussis (IgG, IgM, IgA)
- Tetanus Toxoid (IgG, IgM, IgA)
- Hepatitis B (IgG, IgM, IgA)
- Herpes Type 6 (IgG, IgM, IgA)
- Clostridium Neurotoxin (IgG, IgM, IgA)

NEURO-AUTOIMMUNEANTIBODIES INDUCED BY DIETARY PROTEINS AND INFECTIOUS AGENTS

- Myelin Basic Protein (IgG, IgM, IgA)
- Neurofilament (IgG, IgM, IgA)
- Milk Butyrophilin (IgG, IgM, IgA)
- Streptococcus M Protein (IgG, IgM, IgA)
- Chlamydia pneumoniae (IgG, IgM, IgA)

AUTOIMMUNE REACTION AND INVOLVEMENT OF METALS

- Mercury (IgG, IgM, IgA)
- Fibrillarin (IgG, IgM, IgA)
- Chromatin (IgG, IgM, IgA)
- Immune Complexes (IgG, IgM, IgA)
- Metallothionein (IgG, IgM, IgA)

NEUROTRANSMITTERS AND ANTIBODIES

- Serotonin Receptor Antibodies (IgG, IgM, IgA)
- Serotonin Antibodies (IgG, IgM, IgA)

- Somatostatin Antibodies (IgG, IgM, IgA)
- Vasoactive Intestinal Peptide (IgG, IgM, IgA)
- Prodynorphin + Dynorphin (IgG, IgM, IgA)
- Dipeptidylpeptidase IV (CD26) (IgG, IgM, IgA)

[0111] The disclosure below is of specific examples setting forth preferred methods for embodiments. These examples are not intended to limit the scope, but rather exemplify preferred embodiments.

EXAMPLE 1

Antibodies to neuron-specific antigens in children with autism: possible cross-reaction with encephalitogenic proteins from milk, *Chlamydia pneumoniae*, and Streptococcus group A

[0112] We detected antibodies against nine different neuron-specific antigens in the sera of children with autism. These antibodies were found to bind with different encephalitogenic molecules, which have sequence homologies with neurological antigens (butyrophilin, a milk protein; *Chlamydia pneumoniae* peptide; and Streptococcus M proteins). Our results suggest a role for antibodies against brain cross-reactive food antigens and infectious agents in the pathogenesis of autistic behavior.

Materials and Methods

Patients

[0113] Forty subjects (23 males and 17 females) 3 to 12 years of age (mean 6.4 years), with a diagnosis of autism were sent by different clinicians to our laboratory for immunological examination. The clinical diagnosis of autism was made according to the DSM-III-R criteria, established by the American Psychiatric Association, Washington D.C., as well as by a developmental pediatrician, a pediatric neurologist, and/or a licensed psychologist. Blood samples were excluded if their medical histories included head injury, evidence of gliomas, failure to thrive, and other known factors that may contribute to abnormal developmental. For comparison, blood samples from forty healthy, age and sex matched controls were included in this study.

Neuronal and other Antigens

[0114] Myelin basic protein, myelin associated glycoprotein, ganglioside GM₁, α , β -crystallin, sulfatide, chondroitin sulfate and tubulin were purchased from Sigma Chemicals (St. Louis, Missouri). Neurofilament (NAFT) was purchased from Boehringer Mannheim Roche (Indianapolis, Indiana). MBP peptide 87-106, MOG peptides 21-40, 61-80, milk butyrophilin peptide 89-109, Streptococcal M6 peptide, and *Chlamydia pneumoniae* peptide 483 bound to KLH were purchased from Research Genetics (Huntsville, Alabama).

ELISA Procedure

[0115] Enzyme-linked immunosorbent assay (ELISA) was used according to the above procedures.

Results

Detection of neurologic antibodies

[0116] Using ELISA assays, sera from 40 healthy subjects and 40 autistic children were analyzed for the presence of IgG, IgM, and IgA antibodies against neuron-specific antigens and three encephalitogenic and cross-reactive proteins. The ELISA results expressed as mean O.D. at 492 nm are summarized in Fig. 7. The O.D. for IgG antibody values obtained with 1:100 dilution of healthy control sera ranged from 0.01 to 0.84, varying among subjects and antigens. The mean \pm standard deviation (S.D.) of these O.D. values as shown in Fig. 9 ranged from 0.13 ± 0.09 to 0.23 ± 0.18 . The corresponding IgG O.D. values from autistic children's sera ranged from 0.05 to 2.47 and with the mean \pm S.D. of IgG values, which ranged from 0.41 ± 0.33 to 0.72 ± 0.65 (Fig. 7). For all 12 antigens, the differences between mean \pm S.D. of control sera and autistic children's sera were highly significant ($p < 0.001$). At a cutoff value of 0.3 O.D., levels of IgG antibody against these antigens were calculated in control and patient's sera and found that while 5-22.5% of control sera had IgG values higher than 0.3 O.D., the autistic children's group showed elevated IgG values from 47.5 to 57.5% ($p < 0.001$) (Fig. 8).

[0117] Levels of IgM antineuron-specific antigens in sera of healthy controls and autistic children are shown in Fig. 9. These serum IgM antibodies against all 12 different tested antigens were significantly higher in patients than in controls. The mean \pm S.D. for

controls ranged from 0.12 ± 0.13 to 0.22 ± 0.23 O.D. and for patients ranged from 0.43 ± 0.32 to 0.92 ± 0.63 OD ($p < 0.001$) (Fig.9). When the 0.3 O.D. cutoff point was used, 10 to 20% of controls versus 57.5 to 72.55 % of autistic children's sera showed elevated IgM antibody levels ($p < 0.001$) (Fig. 10). Likewise, IgA antibody levels against these neurological antigens were examined in both groups. Individual and mean \pm S.D. data depicted in Fig. 12 showed significant differences between control and patients groups. The mean \pm S.D. for IgA antibody levels in controls ranged from 0.10 ± 0.07 to 0.2 ± 0.22 and in patients, from 0.25 ± 0.28 to 0.53 ± 0.52 (Fig. 11) ($p < 0.001$). Percent elevated serum IgA anti-neuronal autoantibodies at the O.D. value of greater than 0.3, were significantly higher in autistic children than in controls. The percent positive for IgA antibodies in controls ranged from 5 to 15% and in patients 20-52.5% ($p < 0.001$) (Fig. 12).

Discussion

[0118] Indeed when we tested IgG, IgM, and IgA antibodies against these three peptides, we found that every single serum with ELISA values higher than 0.3 O.D. against neurological antigens exhibited high levels of antibodies against Streptococcal, *C. pneumoniae* and milk peptides as well (Fig. 9-12). Overall, antibodies against these three peptides (first IgM then IgG) were elevated in a higher percentage of controls and experimental sera than the percentage of elevated antibodies against neurological antigens. But, we did not observe even one specimen with a high antibody level against these peptides without having antibody levels against one or all nine tested neuron-specific antigens. These antibodies appear to be specific since in our absorption studies, milk butyrophilin, *C. pneumoniae* and Streptococcal peptide had a similar effect to MBP or MOG in reducing antibody levels from highly positive sera. Based on these findings, we postulate that dietary and infectious antigens play a role in the pathophysiology of autism. It is likely that environmental factors including infection-induced injury causes release of neuronal antigens, which through activation of inflammatory cells, could lead to autoimmune reactions in genetically susceptible individuals. However, only long-term studies can prove the protective versus pathogenic role of these antibodies in children with autism.

EXAMPLE 2

Infections, toxic chemicals and dietary peptides binding to lymphocytes receptors and tissue enzymes are major instigators of autoimmunity in autism

[0119] Based on observations and since so little is known about the range of intestinal immune functions that are shaped by dietary proteins, xenobiotics and infectious agents in autism, we decided to test the hypothesis that infectious agent antigens, dietary peptides and haptenic chemicals may bind to DPP IV (CD 26) and CD69, resulting in autoantibody production and modulation and expression of immune and inflammatory reaction in autism.

MATERIAL AND METHODS

Patients

[0120] Blood samples from fifty subjects (33 males and 17 females), 3-14 years of age (mean 7.2 years), with a diagnosis of autism, were sent by different clinicians to our laboratory for immunological examination. Clinical diagnosis of autism was made according to the DSM-III-R criteria, established by the American Psychiatric Association (Washington, DC) as well as by a developmental pediatrician, a pediatric neurologist, and/or a licensed psychologist. Samples were excluded if their medical histories included head injury, evidence of gliomas, failure to thrive, and other conditions that may contribute to abnormal development.

[0121] For comparison, serum samples from 50 healthy matched controls with negative anti-nuclear antibody titers and no known autoimmune diseases were include. The test requests were properly documented and kept in a confidential file. All persons gave their informed consent and allowed including of their data in this manuscript without disclosure of their identity in the publication.

Patient, Proteins and Reagents

[0122] Gliadin peptides QQLPQPQQPQQSFPQQQPF, LQLQFPQPQLPYPQPQLPY - P Q P L P Y P Q P Q P F, QQPQQFZPQQPYYPZXPZLGZZZPFPPZ, gluteomorphin ZGZPGYYPTSPZZPGQEQ, casomorphin ZTZSLVYPFPGPIPNSLP, B-casein LHLPLPLLZSWMHZPHZPL and CD69

antibody binding epitope MECEKNLYWICNKPYK were synthesized by Bio-Synthesis Inc. (Lewisville, TX). Dipeptidylpeptidase IV (CD26), streptokinase (SK), lipopolysaccharide (LPS), human serum albumin (HSA), mercury [o-carboxyphenyl] Thio] ethyl mercury sodium salt (Thimerosal) were purchased from Sigma (St. Louis, MO).

Binding of Thimerosal to Human Serum Albumin

[0123] For this preparation, 100 mg of human serum albumin (HSA) was dissolved in 9 ml of buffer solution containing potassium chloride and sodium borate 0.05 ml/liter and pH was adjusted to 9.4 with 0.1 N NaOH. Then 25 mg of Thimerosal or sodium merthiolate was dissolved in one ml of H₂O and added dropwise to the HSA solution while stirring over a period of one hour. The reaction mixture was stirred overnight, dialyzed against 0.1 M PBS using tubing with a cutoff of 8000 Dalton. Conjugation of ethyl mercury to HSA was confirmed by SDS gel electrophoresis (shift in the HSA band). In addition spectrograph analysis of conjugate was undertaken. There was a marked increase in absorption from 230 to 260 nm, which indicated that ethyl mercury became covalently linked to the protein carrier (HSA).

Antibodies

[0124] Antibodies to CD26 and CD69 were prepared in rabbits according to standard protocols by Biosynthesis (Lewisville, TX). These polyclonal antibodies were purified by affinity chromatography on protein A-sepharose first and then labeled with horseradish peroxidase.

ELISA Procedure

[0125] Enzyme-linked immunosorbent assay (ELISA) was used according to the above procedures.

Results

Anti-CD26 and CD69

[0126] We investigated whether autoantibodies to CD26 exist in the sera of patients with autism by ELISA using highly purified CD26. As shown in Fig. 13, at a cutoff of 0.3 O.D. or 2 S.D. above the mean and sera dilution of 1:100, IgG, IgM and IgA isotype anti-CD26 autoantibodies were detected in 24 of 50 (48%) for IgG, 20 of 50 (40%) for IgM.

and 22 of 50 (44%) for IgA in patient serum samples. In contrast, autoantibodies to CD26 were detected in 14%, 10% and 8% of healthy donors. The mean \pm S.D. for these antibodies in controls ranged from 0.13 ± 0.13 to 0.15 ± 0.14 and in patients, significantly elevated and ranged from 0.34 ± 0.27 to 0.41 ± 0.39 with p-value being highly significant ($p < 0.0001$). Each serum sample was also tested for the presence of anti-CD69 autoantibodies by using the specific CD69 epitope. Analysis of anti-CD69 IgG, IgM and IgA levels in controls and patients with autism showed significant differences between antibody values and % elevation of these antibodies against CD69 (Fig. 13). The mean \pm S.D. of O.D. values in controls ranged from 0.09 ± 0.09 to 0.11 ± 0.09 and for patients, from 0.27 ± 0.21 to 0.45 ± 0.44 ($p < 0.0001$). Similar to antibodies against CD26, these values for CD69 were the highest for IgA, and then for IgG or IgM levels.

[0127] Eight of 50 (16%) or 7 of 50 (14%) of patients showed simultaneous elevation in IgG, IgM and IgA antibodies against CD26 and CD69. This simultaneous elevation of antibodies was not detected in sera of any of the healthy controls (Fig. 13).

Antibodies Against Gluten and Casein Peptides

[0128] Having shown that a subpopulation of children with autism exhibited antibodies against CD26 and CD69, we then set out to show that these antibodies are generated in response to dietary peptides, infectious agent antigens (SK) and ethyl mercury. Using similar ELISA methods, the results of IgG, IgM and IgA antibodies against gluten peptides are shown in Fig. 14. The O.D. for IgG antibody values with 1:100 dilutions of healthy control sera ranged from 0.01 - 0.84, varying among subjects. The mean \pm S.D. value were 0.17 ± 0.17 . The corresponding IgG O.D. values from autistic children's sera ranged from 0.03 - 1.18 with a mean \pm S.D. of 0.34 ± 0.29 . At a cutoff value of 0.3 O.D., levels of IgG antibody against gliadin peptides were calculated and found that while six of 50 (12%) of controls had high IgG values, patients showed IgG elevation in 22 or 44% ($p < 0.0001$). Levels of IgM and IgA anti-gliadin peptides in controls and children with autism are also shown in Fig. 14. Similar to IgG, at 2 S.D. above the mean, these antibodies were significantly higher in patients, 36% for IgM and 46% for IgA, while in controls, 10% were elevated for IgM and 12% for IgA ($p < 0.0001$).

[0129] In conjunction with the increase of IgG, IgM and IgA antibodies against gliadin peptides, we observed a statistically significant increase of anti-casein peptide antibodies in patients' sera. The mean \pm S.D. of antibodies against casein peptide for controls was 0.16 ± 0.17 for IgG, 0.16 ± 0.13 for IgM and 0.14 ± 0.09 for IgA antibodies.

[0130] The corresponding values in patients with autism were 0.39 ± 0.38 for IgG, 0.40 ± 0.41 for IgM, and the highest value, 0.52 ± 0.52 for IgA antibodies (Fig. 14). Percent elevation of IgG, IgM and IgA antibodies in controls were 10%, 8% and 8%, while 42%, 34% and 42% of patients' sera at the cutoff of 0.3 O.D. showed IgG, IgM or IgA antibodies against casein peptides.

Anti-Streptokinase (SK) Antibody Levels

[0131] Analysis of anti-SK IgG, IgM and IgA levels (Fig. 15) shows that while only one or two out of 50 control specimens (2-4%) had elevated antibodies, a significant percent of patients (18%, 48% and 24%) demonstrated IgG, IgM or IgA elevation. The mean \pm S.D. of anti-SK antibodies was significantly elevated in patients over controls with IgA and IgM ($p < 0.0001$) and for IgG ($p < 0.008$) (Fig. 15).

Anti-Ethyl Mercury Antibody Level

[0132] Similar to the above determination at a cutoff of 0.30 O.D., levels of IgG, IgM and IgA antibodies against ethyl mercury were calculated in controls and patients' sera and found that while one or two out of 50 (2%-4%) of controls had high IgG values, the patients' group showed IgG elevation in 28% and IgM elevation in 30%. In regards to IgA elevation against mercury, none of the controls and only 5 of 50 patients (10%) had increased antibody levels (Fig. 15). Comparison of these antibody values in controls and patients resulted in p values < 0.0001 for IgG and IgM but < 0.004 for IgA. For this measurement, since ethyl mercury was conjugated to HSA, the O.D. of corresponding wells coated with HSA along were subtracted from the O.D.s of ethyl mercury bound to HSA-coated wells.

[0133] To our knowledge, our analyses are the first to clearly demonstrate that dietary peptides, bacterial toxins and xenobiotics bind to lymphocyte receptors and/or tissue enzymes. This results in autoimmune reactions in children with autism. We suggest that these findings provide a mechanism by which environmental factors modulate the immune

system and should help us develop preventive and therapeutic methods to reduce dietary peptides, bacterial toxins and toxic chemical-induced autoimmune reaction in autism.

EXAMPLE 3

Heat Shock Protein and Gliadin Peptide Promote Development of Peptidase Antibodies in Children with Autism and Patients with Autoimmune Disease

[0134] We assessed a hypothesis in a group of healthy control subjects compared to patients with autism and patients with mixed connective tissue disease. Our data suggests a potential role for HSP-60 and dietary peptides in this process.

Materials and Methods

Patients

[0135] Blood samples from fifty subjects (33 males and 17 females), 3-14 years of age (mean 7.2 years), with a diagnosis of autism, were sent by different clinicians to our laboratory for immunological examination. The clinical diagnosis of autism was made according to the DSM-III-R criteria, established by the American Psychiatric Association (Washington, DC), as well as by a developmental pediatrician, a pediatric neurologist, and/or a licensed psychologist. Samples were excluded if their medical histories included head injury, evidence of gliomas, failure to thrive, and other known factors that may contribute to abnormal development. Blood samples from 50 patients with confirmed diagnosis of mixed connective tissue disease (31 females and 19 males), 36-75 years of age with anti-nuclear antibody (ANA) titer of 640 or greater Sm/RnP speckled pattern chromosome negative were selected from our collection sera preserved at -70°C. For comparison serum samples from 50 healthy (25 children age 3-14, 25 adults age 36-75) controls with negative ANA titers and no known autoimmune diseases were included. The test requests were properly documented and kept in a confidential file. All persons gave their informed consent and allowed inclusion of their data without disclosure of their identity in the publication.

Peptides

[0136] ***Gliadin peptides:*** Gliadin peptide QQLPQPQQPQQSFPQQQPF and *Chlamydia trachomatis* HSP-60 peptide LKQIAAHAGKEGAIIFQQVM, HPLC grade, were synthesized by Bio-Synthesis Inc. (Lewisville, TX).

[0137] ***Proteins:*** DPP IV (CD 26), Aminopeptidase I, Aminopeptidase N (CD13), Streptokinase (SK), Lipopolysaccharide (LPS), Human Serum Albumin (HSA) were purchased from Sigma (St Louis, MO).

[0138] ***Antibodies:*** Antibodies to DPP IV, DPP I, aminopeptidase N, streptokinase, HSP-60, and gliadin peptides were prepared in rabbits according to standard protocols (24) by Cocalico Biologicals, Inc. (Reamstown, PA). These polyclonal antibodies were purified by affinity chromatography on protein A-sepharose (115).

Enzyme-Linked Immunosorbent Assay (ELISA)

[0139] Enzyme-linked immunosorbent assay (ELISA) was used according to the above procedures.

Results

Anti-DPP IV autoantibodies levels in control children with autism and patients with autoimmune disease

[0140] Using ELISA assays, sera from 50 healthy subjects, 50 autistic children and 50 patients with mixed connective tissue disease were analyzed for the presence of IgG, IgM and IgA antibodies against DPP IV. Results expressed as O.D. with mean \pm standard deviation (S.D.) are summarized in Fig. 18. The O.D. for IgG antibody values obtained with 1:200 dilution of healthy control sera ranged from 0.05 – 0.8, varying among subjects. The mean \pm S.D. of these O.D. values ranged from 0.15 ± 0.14 . The corresponding IgG O.D. values from autistic children and patients with autoimmune disease sera ranged from 0.01 – 1.1 and 0.01 – 1.5 with mean \pm S.D. of IgG values which ranged from 0.35 ± 0.28 and 0.43 ± 0.30 . The result of post hoc multivariate comparison tests reported in Table 4 show that while the control groups are statistically alike for IgG, IgM and IgA in anti-DPP IV, both the autism and autoimmune groups were significantly different when compared to the control groups ($p < .001$). At a cutoff value of 0.29 O.D. levels of IgG antibody were calculated in controls and patients' sera and found that while 5 out of 50 (10%) of children controls and 7

out of 50 (14%) of adult controls had high IgG values, the patients' group showed IgG elevation in 54% (autistic children) and 64% (patients with autoimmune disease) ($P < 0.0001$) (Table 3). Levels of IgM anti-DPP IV in healthy controls and patients with autism and autoimmune disease are also shown in Fig. 16. These serum IgM antibodies were significantly higher in patients than in controls. The mean \pm S.D. for controls ranged from 0.14 ± 0.12 and for patients from 0.33 ± 0.26 to 0.37 ± 0.36 ($P < 0.0001$) (Fig. 15). When the 0.29 O.D. cut-off point was used, 8% of controls versus 50% and 46% of patients' sera showed elevated IgM antibody levels ($P < 0.0001$) (Table 3). Likewise, IgA antibody levels against DPP IV were examined in three groups. Individual and mean \pm S.D. data depicted in Fig. 16 showed significant differences between control and patients group. The mean \pm S.D. for IgA antibody levels in controls was 0.14 ± 0.11 and in patients from 0.36 ± 0.33 to 0.51 ± 0.40 ($P < 0.0001$). Percent elevated serum IgA Anti-DPP IV antibodies at O. D. value of greater than 0.29 were significantly higher in patients with autism (44%) and autoimmune disease (58%) than in controls (4 – 6%). (Table 3) Anti-DPP I antibody levels in controls, children with autism and patients with autoimmune disease

[0141] Analysis of anti-DPP I IgG, IgM, and IgA levels in controls and patients with autism or autoimmune disease showed significant differences between the antibody values and % elevation of these antibodies against DPP I (Fig. 17). The mean \pm S.D. of O.D. values in controls were 0.14 ± 0.11 to 0.16 ± 0.15 and, for patients, from 0.30 ± 0.22 to 0.46 ± 0.36 ($P < 0.0001$) (Fig. 17).

Anti-aminopeptidase-N (CD13) autoantibodies levels in controls, children with autism and patients with autoimmune disease

[0142] Similar to the analysis of DPP IV and DPP I data, levels of IgG, IgM and IgA antibodies against CD13 were significantly higher in patients than in controls (Fig. 20). In comparison to DPP IV and DPP I percent elevation of CD13 autoantibodies in patients were significantly lower for IgM ($P < 0.31$) but not for IgG and IgA antibody levels ($P < 0.0001$) (Table 3).

[0143] Table 4 reports that for IgG and IgA, similar to control groups, autoimmune and autism are alike. Yet a significant difference is detected between the control

groups and the autism and autoimmune groups. According to our data, for IgM anti-CD13 no differences between the four groups are detected.

Anti-gliadin and HSP-60 peptides autoantibodies levels in controls, children with autism and patients with autoimmune disease

[0144] Concomitant with the increase of IgG, IgM and IgA against DPP IV, DPP I and CD13, we observed a statistically significant increase of anti-gliadin and anti-HSP-60 antibodies in most patients' sera. Antibodies for controls ranged from $0.14 \pm 0.11 - 0.15 \pm 0.17$ and for patients from $0.36 \pm 0.32 - 0.51 \pm 0.43$ ($P < 0.0001$) (Fig. 19, Table 3). Table 4 reports that IgM and IgA for control groups against Gliadin Peptide are statistically alike. Similarly, autism and autoimmune groups are identical, yet the control groups are different when compared with the autism and autoimmune groups. Finally, Table 4 reports that for IgA against HSP-60 the control groups are statistically not different from the autoimmune and autism groups. But for IgG no similarity between the autoimmune groups and other groups (autism and controls) was detected. The autism and control groups are similar but different from the autoimmune group. For the IgM no differences between the autism group and control-adults were observed, but the autism group was statistically different when compared with control-children. These values as well as % elevation of IgG, IgM, and IgA antibodies against gliadin are presented in Tables 3, 4. For examination of possible involvement of gliadin and HSP-60 peptides in the production of autoantibodies against different peptidases, calculation of simultaneous elevation in these antibodies in patients' sera were made and presented in Figs. 21, 22. Between 57 – 90% of sera from children with autism who had high IgG, IgM or IgA against DPP IV, DPP I or CD13 had simultaneous elevation in these antibodies against gliadin or HSP-60 peptides (Fig. 21). This correlation between IgG, IgM and IgA antibodies against DPP IV, DPP I CD13 and gliadin and HSP-60 peptides in sera of patients with autoimmune disease was from 50 – 100 % (Fig. 22). When concomitant detection of antibodies against all five tested antigens (DPP IV, DPP I, CD13, gliadin peptide, HSP-60) was measured, 72, 30 24 % of sera from children with autism versus 41, 25 and 26 % of sera from patients with autoimmune disease had simultaneous elevation in IgA, IgG, and IgM antibody levels against all tested antigens, respectively.

[0145] Many modifications and variations of the embodiments described herein may be made without departing from the scope, as is apparent to those skilled in the art. The specific embodiments described herein are offered by way of example only. References cited herein are incorporated by reference.

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